Federation Proceedings

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# NOTES ON THE THIRTY-SEVENTH ANNUAL MEETING OF THE FEDERATION

*Chicago, Illinois, April 6–10, 1953*

THE FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL BIOLOGY held its thirty-seventh annual meeting in Chicago, April 6–10, 1953. There was a total registration of 6083, composed of members of the constituent Societies, visiting scientists and representatives of industry in the fields of the Federation, guests, press representatives and exhibitors.

Scientific sessions of the six constituent Societies began on Tuesday morning, April 7 and continued through Friday afternoon, April 10. In all, 1389 papers were presented at 140 sessions and an additional 153 papers were read by title. Of these 522 were in Physiology, 454 in Biochemistry, 281 in Pharmacology, 84 in Pathology, 99 in Nutrition and 102 in Immunology. In addition, eight symposia and panel discussion sessions were held by the Societies, and a special session presented nine motion pictures.

The Joint Session of the Federation was held on Tuesday evening, April 7, in the Grand Ballroom of the Conrad Hilton Hotel. Dr. Vincent du Vigneaud, Chairman of the Federation Board, presided. The three papers presented, on the general topic "Some Aspects of Light and Biology," are printed in this issue of FEDERATION PROCEEDINGS. Another special Federation session took place on Thursday evening, April 9, to present a report on the Survey of Physiological Science currently being made by the American Physiological Society, with support from the National Science Foundation. Dr. R. W. Gerard was Chairman for this session.

The Federation Board and Councils of the constituent Societies met throughout the week, and Society meetings were held for the election of members and officers and the transaction of business. The Pharmacology, Pathology and Nutrition Societies scheduled dinners, and a joint smoker by the Biochemistry and Nutrition Societies was held at the Conrad Hilton on Wednesday evening, April 8. Various other groups also scheduled special meetings, luncheons and dinners during the course of the Federation meeting. Entertainment of women guests not attending the scientific sessions was arranged by a committee headed by Dr. Frances A. Hellebrandt.

Exhibits from 69 industrial firms and 23 exhibits by Society members, institutions and laboratories were displayed in the Exhibit Hall at the Conrad Hilton. The fields represented included books, pharmaceuticals, apparatus and equipment and technical demonstrations.

### PLACEMENT SERVICE

The Placement Service office in Chicago scheduled interviews between employers and applicants and registered candidates for future job openings. Three hundred and fifty-eight candidates were registered, an increase of 26 or 8% over the 1952 registration at the New York City meeting, and 598 interviews were scheduled.

### FUTURE MEETINGS

The next annual meeting of the Federation will be held in Atlantic City, N. J., April 10–16, 1954. In 1955, the Federation will meet in California in mid-April, and in 1956 the convention will return to Atlantic City, April 15–20.

### THE FEDERATION BOARD

For the year beginning July 1, 1953, the American Society for Pharmacology and Experimental Therapeutics will head the Federation and its immediate Past President, Dr. K. K. Chen, will be Chairman of the Federation Board; Dr. C. C. Pfeiffer will be Chairman of the Secretaries' Committee of the Federation. Other Board members for the coming year will be Drs. H. B. Haag (Pharmacology); D. M. Angevine, C. C. Erickson and S. C. Madden (Pathology); C. A. Elvehjem, J. M. Orten and P. L. Day (Nutrition); T. P. Magill, J. Y. Sugg and J. F. Enders (Immunology); E. F. Adolph, H. E. Essex and E. M. Landis (Physiology); D. W. Wilson and Philip Handler (Biochemistry). A third member of the Board from the Biochemistry Society is to be appointed by the Council to fill the vacancy created by the recent death of Dr. Henry A. Mattill. Dr. M. O. Lee was reappointed Federation Secretary for the coming year.

# CORRECTIONS AND ADDITIONS TO MARCH ISSUE

**Page 38.** EDWARDS AND GARBER, *last line of abstract*: "Supported by a grant from A. H. Robins and Company."

**Page 62.** HARRISON AND TALBOT, *last line of abstract:* Add the words, "Supported in part by The Life Insurance Medical Research Fund."

**Page 65.** HENDLEY, HEATH AND HODES, *9th and 10th lines of abstract:* Instead of "5 mA," read "10 V."

**Page 173.** AXELROD AND MILLER, *9th line of abstract:* Instead of "*a*) oxidation . . . in" read "*a*) reduction with the introduction of $C_{11}$ hydroxyl and oxidation with $C_{17}$ hydroxyl groups in. . . ."

**Page 260.** OTTO ROSENTHAL, *7th line of abstract:* The value for $N_w$ should be 2.1.

**Page 275.** CARL M. STEVENS *et al.*, *2nd line of title:* The name of the second author should be PRAN VOHRA. The same correction should be made on page 528 in Program, Part II, and index pages xiv, 592.

**Page 295.** PAUL C. ZAMECNIK, *20th line of abstract:* Instead of "0.24 M $KHCO_3$", read "0.024 M $KHCO_3$."

**Page 300.** The following abstract was omitted:

**Mechanism of action of DFP and TEPP on the patellar reflex.** R. BECK,* E. M. ROBINSON,* B. P. MCNAMARA AND J. H. WILLS. *Pharmacology Branch, Chemical Corps Med. Labs., Army Chemical Center, Md.*

An investigation was conducted in attempt to correlate the effects on the patellar reflex of the anticholinesterase compounds DFP and TEPP and the acetylcholine and cholinesterase levels of the central nervous system. The experiments were performed on cats which were anesthetized with Nembutal, atropinized, and artificially respired. Biopsies of brain tissue were taken *1*) before poisoning, with the knee-jerk functional; *2*) after poisoning, with the knee-jerk absent; *3*) during subsequent recovery of the knee-jerk; and again *4*) after disappearance, and *5*) during recovery produced by a second dose of the anticholinesterase. The acetylcholine and cholinesterase levels of the brain during these 5 periods were determined. The results included a decrease in cholinesterase and an increase in free acetylcholine in *sample 2*, but no significant changes thereafter. These results suggest that the effects of DFP and TEPP on the patellar reflex may involve actions other than that on cholinesterase.

**Page 358.** RADOMSKI AND NELSON, *title* should read: "Toxic effects of synthetic and fermentation chloramphenicol in dogs. Preliminary report."

**Page 425.** HELEN T. PARSONS *et al.*, *last line of abstract:* Instead of "increase," read "decrease."

# JOINT SESSION OF THE FEDERATION

*Chicago, Illinois, April 7, 1953*

## Some Aspects of Light and Biology

*Chairman:* VINCENT DU VIGNEAUD

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# BIOLUMINESCENCE: EVOLUTION AND COMPARATIVE BIOCHEMISTRY[1]

E. NEWTON HARVEY

*From the Department of Biology, Princeton University, Princeton, New Jersey*

IT IS evident that the ability to emit light has been of considerable survival value during evolution in the animal kingdom, if we may judge by the rather extraordinary number of wholly unrelated animals which have developed photogenic organs (1).[2] Luminous species are found in some 40 different groups, from the simplest protozoa to the chordates, where a large number of teleost fish have developed the most perfect lighting system, controlled by nerves or endocrines. A glance at an evolutionary tree will reveal luminous species scattered in about half of the phyla, with no apparent rhyme or reason. Some phyla possess many, others few luminous species. It is possible that all individuals among the Ctenophora are luminous, certainly a very large number emit light. Many of the Cnidaria are luminescent, whereas in the phylum Nemertinea, only one luminous species is known. Among the Mollusca, luminescence is wide-spread in the Cephalopoda, whereas luminous snails, bivalves, and nudibranchs are rare. Thus, in the course of evolution, light production has appeared again and again.

That some slight change in cell processes will result in a light reaction can be inferred from the distribution of luminescence in species of a genus. Among the Dinoflagellata, one species of the genus, Ceratium, is luminous and a closely related species not. The same statement applies to certain genera of higher forms, such as the mollusc, *Pholas dactylus*. In the plant kingdom, a few bacteria and higher fungi are the only light producing forms. Again, one species of a genus may be luminous and another not. Moreover, non-luminous mutants of luminous bacteria occur spontaneously or after irradiation with ultraviolet light. In nature, one species of the fungus, *Panus stipticus*, exists in two varieties, differing only in light production, the non-luminous European strain and the luminous American variety. Hybrids of the two strains are fertile and the ability to luminesce is inherited as a Mendelian dominant, although the genetics is complicated by infertility factors (MACRAE, 1942).

Thus, two important facts are at once apparent: *1*) the wide distribution of bioluminescence in the living world, and *2*) the existence of a luminosity factor whose presence or absence determines whether the organism can emit light or not. The conclusion that light-production must have started from some slight change in a cellular reaction common to all living things is inevitable. The almost universal dependence of light emission on dissolved oxygen suggests that the photogenic system might be connected with the cell respiratory systems, perhaps evolved by a slight change in one of the hydrogen acceptors (HARVEY, 1932). We shall return to this point again.

Once a cell had developed the ability to emit light, perhaps by some mutation involving a necessary reaction, the next complication might

[1] Part of the research on the chemistry of Cypridina luminescence referred to in this paper has been supported by grants to Princeton University from the Research Corporation, New York, and the Office of Naval Research.

[2] An extended review of light production and a complete bibliography will be found in the author's *Bioluminescence* (Academic Press, New York, 1952). Only papers not included in this book or of special import to comparative biochemistry are given in the references to this paper.

be a method of controlling the light. It is universally true that in bacteria and fungi, the light emission is intracellular and the luminescence intensity constant over long time periods, quite independent of stimulation, and changing only with variation in temperature, oxygen pressure, nutrients, drugs, etc. in the medium. On the other hand, in the simplest animal forms, the protozoa, where luminescence is also intracellular and connected with granules in the protoplasm, the light is strictly under control, appearing only on stimulation. It is a response to a stimulus, completely analogous to the excitation of muscle contraction. In the metazoa also, luminescence appears only on stimulation, but in many cases the light is extracellular, from secretion of luminous substances by gland cells. When the light is intracellular, the photogenic cells may be surrounded by accessory structures—lenses, reflectors and pigment screens—so that the luminous organ is actually a lantern, lighted by reflex response to various stimuli. Such organs, spoken of as photophores, are especially complicated among the shrimp, the squid and the fish. Before their light emitting function was known, the photophores were thought to be accessory eyes, so similar is the lens to that of a visual organ.

Perhaps strangest of all, some animals have found light production to be so advantageous that they have developed a symbiotic relation with luminous bacteria and possess a special luminous organ with abundant blood supply to carry the food and oxygen needed by the bacteria. Such relationships are especially common among the fish, where elaborate symbiotic light organs have been developed, including mechanisms for screening the constant luminescence of the bacteria. In the fish, Photoblepharon, this mechanism is like an eyelid, a fold of black skin which can be drawn up over the light organ, whereas in the fish, Anomalops, the whole light organ is hinged and can be turned inward toward the body, thus presenting the black pigmented opaque side outward.

In addition to symbiotic luminous bacteria, and the common saprophytic variety living on dead fish or flesh, there are parasitic luminous bacteria which infect living animals (midges, sand-fleas, caterpillars, etc.), giving them a luminous disease, which is eventually fatal, but which makes the animal while living appear to be a luminous species. In the subsequent discussion on the comparative biochemistry of luminescence, only self-luminous organisms will be considered.

The evolution of complex structures for light emission has always been hard to explain. It is significant that Darwin in the *Origin of Species*, under the heading, "Special Difficulties of the Theory of Natural Selection," placed luminous organs in the same category as the electric organs of fish, and merely said "it is impossible to conceive by what steps these wondrous [electric] organs have been produced." One difficulty was lack of knowledge of the use of luminous organs. The light of the wingless glow-worm was thought to attract the winged male or to frighten enemies. In the *Descent of Man*, in speaking of sexual selection and differences between male and female of a species, Darwin pointed out another use, suggested by Belt, that of a warning signal. The lampyrid beetles, to which the glow-worm belongs, appear to be distasteful to insectivorous mammals and birds. Therefore, "the luminous species profit by being at once recognized as unpalatable . . . It is not known why the wings of the female glow-worm have not been developed; but in her present state she closely resembles a larva, and as larvae are so largely preyed on by many animals, we can understand why she has been rendered so much more luminous and conspicuous than the male; and why the larvae themselves are likewise luminous." Later evolutionists have had little more success than Darwin in visualizing the evolution of luminous organs. Although Goldschmidt (1948) has called attention to adaptation of the New Zealand glow-worm, a discussion of bioluminescence is mostly absent from modern treatments.

The use to the animal of light producing organs is a difficult subject, in many cases a matter of conjecture. In addition to sex attraction, frightening, or warning of predators, the photophores have been considered recognition marks or lures for obtaining food or actual lanterns for seeing. The problem of the use of light by a bacterium, or a fungus, or the myriads of minute organisms floating at the surface of the sea and giving rise to its phosphorescence, is indeed unsolved, if there is a solution.

It is, however, not the evolution of complicated luminous structures or the bionomic significance of light emission that concerns the biochemist but rather the manner of formation by various living cells of a substance which can emit light. There is no doubt that bioluminescence is a chemilumi-

nescence in which some molecule acquires sufficient energy from a chemical reaction to radiate in the visible region. Bioluminescence is allied to fluorescence and phosphorescence, where the energy for visible light emission comes from exciting radiation of some kind. However, innumerable experiments indicate that previous illumination is never necessary for bioluminescence and the only relation between chemiluminescence and fluorescence is a general one. If a compound possesses a molecular structure with electrons which can be readily raised to an excitation level by incident radiation, that is, if it contains fluorophore groups, then these groups are likely to be involved in picking up the energy of a chemical reaction. A fluorescent substance usually turns out to be a chemiluminescent substance, provided the proper reaction is found to supply the free energy necessary for radiation in the visible region. For wave-length 800 to 400 mμ, the quantum equivalent per mol ranges from 35,500 to 71,120 calories, with blue green (500 mμ) = 57,000 calories.

The fundamental chemistry of light production in animals has been known since 1886–87, when the French physiologist, Raphael Dubois, announced the discovery of an oxidizable substance, luciferin, which he believed emitted light when the oxidation was catalysed by the enzyme, luciferase. His experiments were made on the tropical beetle, Pyrophorus, and the mollusc, Pholas. Since that time luciferin and luciferase have been demonstrated in the fire-flies, the ostracod crustacean, Cypridina, the worm, Odontosyllis, the shrimp, Systellaspis, and the fresh water limpet, Latia, but they have not been demonstrated in a very large number of other groups of luminous forms which have been tested, including the bacteria[3] and the fungi. Nevertheless, practically all workers assume the existence of luciferin and luciferase as the primary photogens for light emission.

In the plant kingdom and in almost all animals molecular oxygen is necessary for luminescence, but there are a few exceptions. The Radiolaria and extracts of the medusa, Pelagia, and the ctenophores can emit light in the presence of an excess of sodium hydrosulphite or of hydrogen and finely divided platinum. It is safe to say that no dissolved oxygen whatever is utilized in the luminescent reaction of these forms. The relation between oxygen pressure and light intensity has been studied in a number of organisms by Hastings (3).

In the fire-flies the luminescent system is more complicated. Oxygen is necessary, and, as McElroy (4) and associates have shown, adenosinetriphosphate (ATP) and bivalent cations (Mg) play an important part in light production. It has not yet been demonstrated that ATP and Mg are involved in a number of other groups which have been tested, and it seems quite certain they are not necessary for light production in Cypridina.

The reader will at once notice that there is a considerable diversity in the chemistry of luminous animals, inasmuch as oxygen is essential in some forms, not in others, and luciferin and luciferase can be isolated in some forms, not in others. However, the oxidation of a compound in the presence of an enzyme seems so reasonable a mechanism for light production that the comparative biochemistry of luminescence should certainly start with the question of the structure of luciferin. Is it *a*) one compound in all luminous organisms, or *b*) a group of closely related compounds, substitution products of a particular nucleus, like the sterols, or *c*) are the luciferins of different species wholly unrelated to each other? The discussion will serve to reveal what is known of the properties of luciferin from different species and something regarding the mechanism of luminescence in general.

First it must be pointed out that there is no a priori reason why luciferins should be the same substance or even closely related substances. A survey of the animal kingdom indicates that organisms have generally adopted a great variety of chemicals and chemical reactions in attaining specific ends. Nothing could be more diverse than the means of supplying energy for the organism—from the various photosynthetic systems of plants and the oxidation of proteins, fats and carbohydrates in most animals, to the utilization of sulphur, nitrogen, hydrogen, or iron among the chemoautotrophic bacteria. A similar diversity is to be observed in the final products of chemical change. When nitrogenous waste products of animals are to be excreted, they may be eliminated in the form of ammonia, trimethylamine oxide, urea, uric acid or creatine and creatinine, depending on the environment of the animal. Comparative biochemistry has revealed that the transport of oxygen in the blood may be by

[3] Recently Strehler (2) has described luciferin in bacteria.

hemoglobin, chlorocruorin, haemerythrin or hemocyanin, the latter a completely different type of chemical carrier. The difference in distribution of the phosphagens of muscles, phosphocreatine and phosphoarginine, is a well known example of more nearly related compounds serving the same function, but they are only two among a number of high energy phosphate donors in the living world. Wald (5) has pointed out that when a pigment is needed for light detection or for vision, the function is assumed by the carotenoids, among which a number of different compounds take over the photosensitive function in plant, or flagellate, or man. No single organic

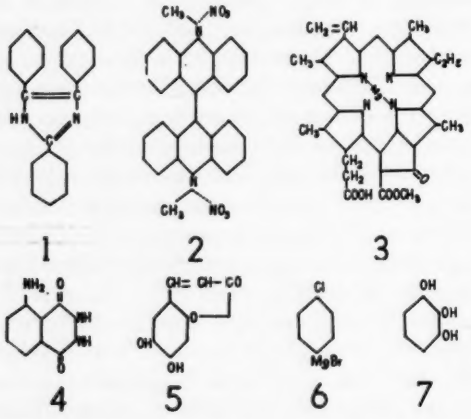


Fig. 1. VARIOUS types of organic chemiluminescent compounds. *1*) Lophin or triphenylglyoxaline; *2*) dimethylbiacridinium nitrate; *3*) a metal porphyrin compound; *4*) aminophthalhydrazide; *5*) aesculetin, present in the glucoside, aesculin; *6*) chlorphenylmagnesium bromide; *7*) pyrogallol. From E. N. HARVEY (1).

compound appears universally to serve a particular purpose throughout the living world. Thus, nature has solved its chemical problems by invoking different rather than the same substance.

Since the ability to luminesce has appeared in the most diverse organisms, from bacteria to fish, why expect that the chemical systems producing light, particularly the photogen, should be the same, or even structurally related in the different groups? One might as well reason that all fluorescent substances observed in organisms must belong to the same chemical class, or to claim that all yellow pigments should be related to each other.

Second, there are some general arguments which point to the supposition that the luciferins of luminous organisms could be widely different in chemical constitution. One line of evidence is drawn from a study of the emission spectra of various chemiluminescent organic compounds whose molecular structure is well known; see Anderson's review (6). It has been found that totally unrelated substances, whose emission lies in the visible region, may exhibit maximum wave-lengths widely different from each other, whereas closely related compounds have maximum wave-lengths near together. The best known chemiluminescent substances are illustrated in figure 1. Aminophthalhydrazid (luminol) has a blue chemiluminescence, dimethylbiacridinium nitrate (lucigenin) a green chemiluminescence, pyrogallol a yellowish and metal porphyrin complexes a red chemiluminescence. Bioluminescent spectra of different groups of animals have the same wide distribution of maximum emission, from the red light of the railroad worm, the yellow of some fire-flies and the green of luminous bacteria to the blue of Cypridina. Certainly the luciferins of these generally unrelated groups could be fundamentally different in structure.

When the emission spectra of a group of related compounds, for example the phthalhydrazids (or dihydrophthalazinediones), are studied, it is found that substitution of various groups, such as $NH_2$, OH, Cl etc., in the 5 position does change not only the luminescence intensity but also the maximum wave-length of emission. However, the spectral maximum only changes to a relatively slight extent, for example from bluish violet to greenish blue, as recorded by eye observation by Drew and Pearman (7). No substitution product emitted red, orange or yellow light, with the exception of hydromellitaz-1-4-6-9-tetraone, a bicyclophthalhydrazide of quite different structure, made up of three rings instead of two. Its chemiluminescence was yellow. Some of the color differences could have been due to the formation of colored reaction products, whose absorption modifies the true chemiluminescent spectrum. The spectral energy curves of 10 of the phthalhydrazids with different substituted groups in position 5 have been measured by Spruit-van der Burg (8) who obtained maxima which varied from 402 to 462 m$\mu$, while the luminescence intensities varied 10,000-fold.

Similar slight changes in spectral maxima are to be found among diacridyl derivatives. Dimethylbiacridinium nitrate, often called lucigenin, emits a yellow green chemiluminescence like that of some fire-flies, when treated with

$H_2O_2$ and a catalyst. Substitution in the bi-acridinium nucleus changes the spectrum of both the chemiluminescence and fluorescence of the lucigenins; see Gleu and Schaarschmidt (9), again, however, in the ranges blue to green. The interpretation of these spectra is somewhat difficult, as it has been shown by these workers, as well as by Kautsky and Kaiser (10), that the spectrum depends on concentration of the lucigenin and the $H_2O_2$, and is actually made up of a blue chemiluminescence and a green fluorescence excited by the chemiluminescence, the resulting spectral maximum depending on whether fluorescence or chemiluminescence contributes most to the final light emission.

*phosphoreum* when the population density of the suspension was reduced to $\frac{1}{20}$. Using dilute suspensions of various species, the maxima were found to vary from 472 m$\mu$ for *Ph. phosphoreum*, 489 m$\mu$ for *Ph. splendidium*, to 496 m$\mu$ for *Ph. fisheri*, while the maxima for three fungi, *Mycena polygramma*, *Omphalia flavida* and *Armillaria mellea* (VAN DER BURG, 1943) was 526 m$\mu$. These spectrophotometric curves, shown in figure 2, are undoubtedly the most accurate thus far obtained. They exhibit only one maximum but are somewhat asymmetrical.

Three fungi investigated by Haneda (1942) showed maxima at 512, 516, and 516 m$\mu$, respectively. The work of Coblentz (1912) with four

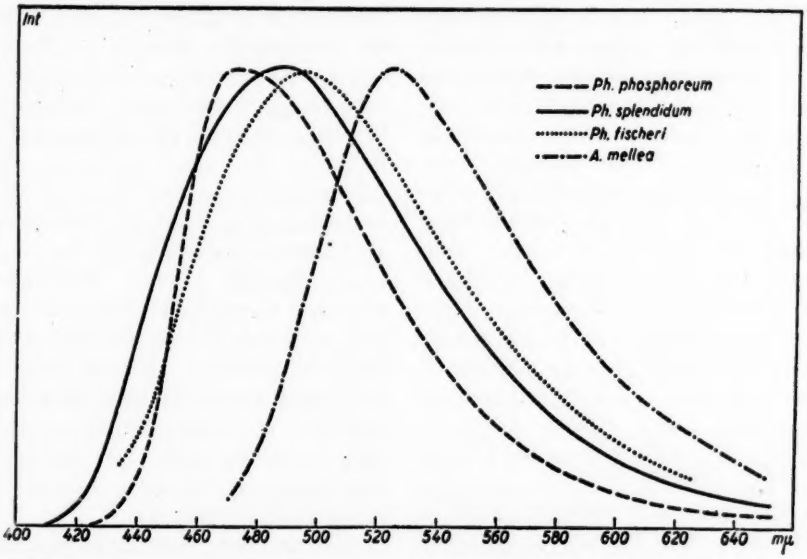


Fig. 2. EMISSION SPECTRA of various species of Photobacterium and the fungus *Armillaria mellea*. From A. SPRUIT-VAN DER BURG (11).

As far as is known, all metallic porphyrin derivatives, if they luminesce when oxidized, show red chemiluminescence, whereas lophin, amarin and related substances show a bluish chemiluminescence. The emission maxima may vary slightly but not much.

Slight changes in the spectral distribution curve of chemiluminescent substances are to be compared to differences in the spectra of 3 different species of luminous bacteria studied by Spruit-van der Burg (11) in very dilute suspensions, where pigments or fluorescent substances in the bacteria would have a minimum effect on spectral distribution. These observers noted a shift connected with absorption from about 500 to 472 m$\mu$ in the maximum emission of *Photobacterium* different species of fire-flies indicated a range of maxima from 552–580 m$\mu$. Thus the variation of spectral maxima among different luminous bacteria and fungi is about the same as among the various substitution products of a group of chemiluminescent substances. The same is true for the spectra of various fire-flies.

The most interesting animals from the point of view of biochemistry are those which emit light of two colors. Among these, the best known is the railroad worm, Phrixothrix, of South America, related to the fire-flies. The insect displays a row of yellowish green lights along the side of the segments and a vivid red light in the head. The red color is not due to a pigment screen, and there is no red fluorescence of the

head organ, whose tissues are colorless to eye observation. The author finds it difficult to believe that some porphyrin compound is not involved in the red light emission but there is as yet no evidence for this view (HARVEY, 1944, 1945). The animal is so scarce that extended biochemical studies have not yet been attempted.

Perhaps a better criterion of chemical relationship among photogenic substances than spectral emission is the luminescence after interaction of luciferin and luciferase in extracts of different luminous organisms. When the luciferin of one species is mixed with the luciferase of another species, light will result if the animals are closely related—different species of fire-flies (orange to yellow light) or different species of ostracods (bluish light)—but no light appears if luciferin from a fire-fly is mixed with luciferase of an ostracod, or vice versa. Among other groups in which the luciferin-luciferase reaction has been demonstrated (shrimp with external luminous secretion; the mollusc, Pholas, and the worm, Odontosyllis), no light has been obtained when luciferin (or luciferase) from any of these forms has been mixed with luciferase (or luciferin) from the crustacean, Cypridina. Scarcity of material has prevented a complete investigation of the results of intermixing luciferin and luciferase from all known luminous species, but the evidence at hand indicates definite specificity unless the organisms are very closely related.

Such behavior may very well reflect a great difference in constitution of what has been called 'luciferin' in the animals of different groups, a difference so great that the enzyme-substrate complex (luciferase—luciferase compound), an intermediary step in the reactions leading to light emission, cannot be formed. At one time the author compared the luciferins to the hemoglobins of different animals, which differ in crystal characteristics and protein specificity, but evidence is accumulating to indicate that a far better comparison is between structurally unrelated chemiluminescent molecules in certain great groups of light producing animals, with a variety of substituted derivatives among the various families, genera, or species of the groups. Such a situation does not exclude the additional possibility of protein specificity among the luciferases.

Unfortunately the actual chemical structure of a luciferin is not known with certainty for any luminous organism. In his last paper, Dubois (1928) concluded that Pholas luciferin exhibited the properties of "albumines naturelles," while the author in an early work (1919) designated Cypridina luciferin a proteose. Other suggestions for luciferins from various authors have included phospholipids, flavins, polyhydroxyphenols and a naphthohydroquinon structure, especially one with a ketohydroxy side chain.

Extensive chemical investigations have been carried out only with the bacteria (12), with Cypridina (13), and with fire-flies (4). Luminous bacterial research has been chiefly on oxidative systems of the whole cell since all attempts to isolate and purify photogenic substances from these organisms have failed. A large amount of information on the luminescence of bacteria in relation to salts, nutrients, drugs, temperature, pressure, radiation, etc. has been accumulated in the last eighteen years by F. H. Johnson, H. Eyring and co-workers in Princeton, N. J., by A. J. Kluyver and many colleagues in Delft, Holland, and by W. D. McElroy and students in Baltimore, Md.

In recent years, the chemistry of Cypridina luciferin and luciferase has been largely studied at Princeton University by Harvey, Anderson, Chase, Rexford, Mason, and Tsugi, while the chemistry of fire-fly luciferin and luciferase has been attacked at Johns Hopkins University by McElroy, Strehler, Hastings and Coulombre. Although the materials have not been completely purified and neither luciferin nor luciferase has been crystallized, enough of the properties have been determined to make a comparison of the luciferins significant.

First, it must be again emphasized that the Cypridina luminescence system requires only luciferin, luciferase and oxygen in aqueous solution in order that light may be emitted. No salts are necessary; in fact light is produced when a chelating agent (versene) is added to the luciferin-luciferase mixture. Anions and cations do, however, affect the intensity of the luminescence.

The fire-fly luminescence system requires luciferin, luciferase, adenosinetriphosphate (ATP), magnesium ions and oxygen in aqueous solution for light production. No light appears if any of these 5 factors is absent. If any 4 of the factors are present, it is found that the light intensity on adding the 5th factor is proportional to the concentration of the 5th factor, up to a certain limit, with the exception of luciferase addition. No limit on light intensity has yet been observed when various concentrations of luciferase are added to luciferin + ATP + Mg + $O_2$.

In requiring ATP for luminescence, the fireflies (and related beetles like Pyrophorus) differ strikingly from Cypridina and a number of other luminous groups. A survey by Harvey and Haneda (14) has indicated that ATP added to luminous extracts of 7 groups of luminous animals (unrelated to beetles) did not in any case revive the luminescence. Recently 7 additional groups have been tested with ATP and found to behave in the same way. Although a negative ATP reaction is not a certain indication (see McElroy and Harvey, 15) that the luminescent system functions without ATP, the evidence thus far accumulated suggests that the fire-fly system may be unique.

Perhaps the most important property of luciferins for diagnostic purposes is the absorption spectrum, a distinctive characteristic of groups of organic compounds. Changes during oxidation are to be expected, since many redox systems are colorless in one state (usually the reduced), colored in the other. It was early observed that Cypridina luciferin oxidizes in two ways—with light emission in presence of luciferase, and spontaneously without light emission in absence of luciferase. The colorless spontaneously oxidized luciferin will give no light with luciferase, but can be reduced by hydrogenation methods, if not allowed to stand for any length of time, and will then luminesce with luciferase. The redox potential is slightly more negative than quinhydrone. Oxidation of luciferin in presence of luciferase is much more rapid, and yields an oxidation product, also colorless, which cannot be reduced, even if the hydrogenation is attempted immediately.

The absorption curve for the purest[4] Cypridina luciferin has been recorded by Chase and Brigham (16) in the ultraviolet and visible. As shown in figure 3, at a pH = 6.8 there is a sharp maximum absorption peak at 265 mμ, a shoulder at 310 mμ and a broad maximum at 435 mμ. During oxidation in the air, the extinction at 265 mμ decreases about ⅓ to a stable value, while simultaneously a new maximum appears at 365 mμ, and the 435 mμ absorption band is replaced by one at 470 mμ which subsequently disappears. Coincident with the decrease in extinction of the 265 mμ band and the appearance of the new band at 365 mμ, the luciferin loses its ability to produce light with luciferase. In the visible region the changes in the spectrum (replacement of 435 mμ by 470 mμ and disappearance of 470 mμ) also take place during light production by luciferase, i.e., no difference in spectral changes can be detected during dark and luminescent oxidation. Ultraviolet absorption of the protein luciferase has so far interfered with corresponding measurements in the 265 mμ region.

McElroy and coworkers (17) have recorded the absorption spectrum of the purest fire-fly luciferin. At pH = 7, there is a major absorption peak at 330 mμ and a secondary peak at 263 mμ. No changes during oxidation have been reported. The first absorption peak (330 mμ) results in fluorescence of the fire-fly luciferin in ultraviolet light with a yellow green color in acid and a pale red fluorescence in alkaline solution. The absorption spectra are such as to make the color of fire-fly luciferin yellowish in alkaline, colorless in acid solutions, the color change indicating a pK = 8.3, which also corresponds to a change in the fluorescence spectrum in acid and alkali. Cypridina luciferin, on the other hand, is yellow in acid and practically colorless in alkaline solution (Chase, unpublished observations) and exhibits no marked fluorescence in ultraviolet light in water at any pH or in solution in any of the alcohols.

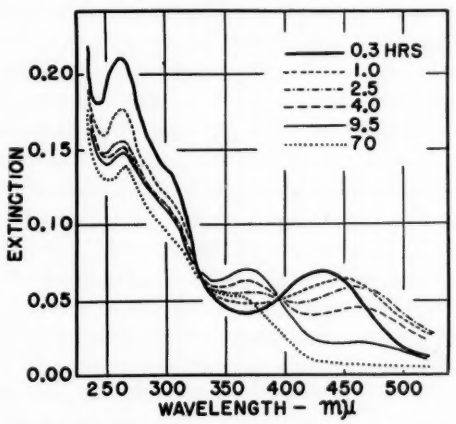


Fig. 3. Absorption spectra of a Cypridina luciferin solution in 0.067 M phosphate buffer at pH 6.8, measured after exposure to air for the time intervals indicated. After A. M. Chase and E. H. Brigham (16).

No corresponding absorption curves have been obtained for bacterial luciferin since this substance has not been isolated from bacteria. However, Kluyver, van der Kerk and van der

[4] All the properties of luciferins are based on the purest preparations thus far obtained, by Anderson's (27) extraction procedure.

Burg (18) studied the luminescence inactivation spectrum of *Photobacterium phosphoreum* in ultraviolet and visible light, i.e., the relative effectiveness of different wave lengths in suppressing luminescence. Their curve should represent the absorption spectrum of some substance essential to the luminescence reaction. Van der Kerk regarded the substance affected by the light as bacterial oxidized luciferin but Spruit (19) concluded that bacterial luciferin was the substance affected, and pointed out that the inactivation spectrum agreed well with the absorption spectrum of 1-4 dihydroxy-naphthyl-2-hydroxymethylketone, that is the naphthohydroquinon with a CO—$CH_2OH$ group substituted in the 2 position. According to Chase and Brigham (16), the absorption spectrum of Cypridina luciferin does not correspond to the above compound, nor does this compound emit light when mixed with Cypridina luciferase (20, 21).

The solubilities of fire-fly and Cypridina luciferin are not too different, both being soluble in water over a wide pH range, soluble in the alcohols, at least as far as amyl alcohol, but insoluble in benzene, petroleum ether and ethyl ether. Both are stable on heating in water, the fire-fly in presence of oxygen and Cypridina luciferin only in absence of oxygen, since it is readily oxidized in water solution, even at room temperature, especially in alkaline reaction. Fire-fly luciferin is destroyed on heating in 1 N HCl or 1 N NaOH for one hour, whereas Cypridina luciferin is stable under these conditions provided oxygen is absent. In 4 N HCl at 125° C, Cypridina luciferin is slowly decomposed with loss of ability to luminesce. Both luciferins are stable on drying.

Elementary analysis indicates C, H, O, and N present in Cypridina luciferin but no S, P, or halide detectable by standard procedures.[5] Zn has occurred in certain samples but its presence in the luciferin molecule is uncertain. The corresponding analyses for fire-fly luciferin have not been made.

The recent work of Mason (22) and Mason and Davis (23) in the Princeton laboratory indicates that Cypridina luciferin may exist in an alpha and beta form, both capable of luminescence, but with different chromatographic behavior. The alpha is converted to the beta form on heating at 65° in high vacuum, a transformation reversed by dilute acid. Infra red absorption studies of beta-luciferin films show absorption peaks at 3250, 2825, 1680, 1625 and 1510 $cm^{-1}$, which indicate the amide bond as it occurs in peptides or cyclic ureides. Yellow beta-luciferin itself does not respond to the ninhydrin test but when it is hydrolysed in acid, the amino acids, glycine, threonine, proline, lysine, aspartic acid, glutamic acid, and either leucine, isoleucine or phenylalanine, together with a yellow pigment (not luminescent with luciferase) can be identified by two-dimensional paper chromatography. The above behavior leads to the view that Cypridina luciferin is a polypeptide with a closed ring, containing a yellow pigment, i.e., a chromopolypeptide. Luminescent activity is associated with such a compound, but also present together with the active polypeptide is a colorless polypeptide (probably oxidized luciferin) which gives the same ammo-acids on hydrolysis, but no light if mixed with luciferase. Recently Tsuji, Williams and Wooton (unpublished observations), using the quantitative exchange resin technique of Moore and Stein (24), have identified at least 5 more amino acids[5] in purified luciferin preparations. Corresponding tests for amino acids have not yet been made with fire-fly luciferin.

Thus, in spectral absorption, lack of marked fluorescence, ready oxidation, color changes in acid and alkali and ability to luminesce without Mg or ATP, Cypridina luciferin differs markedly from luciferin of the fire-fly.

Returning now to the question of the relation of the luciferin-luciferase system to essential enzyme systems in cells, especially the possibility that luciferin is a modified precursor of some prosthetic enzyme group, the flavins appear to have been first considered by Eymers and van Schouwenburg (1937) in the case of luminous bacteria. A more definite designation of flavins as the basis of luminescence has been made by Backovsky, Komarek and Wenig (1939) for earthworms. The Czech workers (Wenig, 25; Wenig and Kubista, 26) have demonstrated the presence of riboflavin (not flavin phosphate or flavin adenine dinucleotide) in the yellow lymphocytes of the luminous earthworm, Eisenia submontana, as well as in those of a non-luminous form, Eisenia foetida. The luminous lymph of

[5] The sulphur-containing amino-acid, methionine (not cystein or cystin) appears to be present in such small quantity that elementary analysis reveals no sulphur.

E. submontana fluoresces yellow green like riboflavin until the bioluminescence has disappeared, at which time the fluorescence color changes to blue, that of lumiflavin. A corresponding change in the yellow green fluorescence of the non-luminous lymph of E. foetida does not take place. Consequently the Czech workers have postulated that the bioluminescence of the earthworm is connected with a change from riboflavin to lumiflavin, a reaction which does not occur in the non-luminous species. The molecules of riboflavin are believed to be "adsorbed in an oriented layer on the surface of granula of lipoid character. . . . [and] the activation of energy which brings them into an excited state is probably derived from an oxidative reaction in which molecular oxygen takes part."

It is interesting to note that the luminous granules of the earthworm are yellow in color. The luciferin of Cypridina is also yellow (although its luminescence is blue), and a yellow color has been observed associated with luminous cells in at least seven additional groups—Hydromedusae, Polychaeta (Tompteridae and Terebellidae), Nudibranchia, Copepoda, Chilopoda, Lampyrid fire-flies and Macruroid fish. If bioluminescence has been evolved in connection with cell respiratory systems the yellow flavins immediately become suspect photogens. They are widely distributed, are fluorescent and can exist in reduced and oxidized form. Flavins are the prosthetic groups of the yellow respiratory enzymes, a relationship possibly comparable to the luciferin-luciferase complex. However, the free energy of riboflavin oxidation is only about 45,000 calories, insufficient for radiation in the blue region.

It seems quite certain that neither Cypridina luciferin nor fire-fly luciferin are riboflavins. The lantern of the fire-fly does contain unusually large amounts of riboflavin (Brooks, 1940; Ball and Ramsdell, 1944) but the luciferin absorption spectra are quite different and other properties rule out riboflavin as the light emitting substance in these organisms. During McElroy's (17) purification of fire-fly luciferin, the riboflavins are removed, and the same is true during Anderson's (27) purification of Cypridina luciferin. Reduced or oxidized riboflavin or related compounds will not luminesce when mixed with luciferase (20). However, the possibility that a derivative of the alloxazine nucleus may make up part of the luciferin molecule has not necessarily been ruled out.

Cypridina luciferin is not related to the vitamin A derivatives, the yellow carotenoids, or to vitamin D and E derivatives, the sterols. Thiamine ($B_1$) and biotin nuclei may be eliminated because of the absence or very low sulphur content of luciferin, ascorbic acid (C) because of its lack of nitrogen. Nicotinic acid (PP) and pyridoxine ($B_6$) derivatives (from pyridine), pantothenic acid with a C—NH—CO linkage (a precursor of co-enzyme A) are possibilities. Since luciferin is a yellow compound, various yellow pigments such as the flavins, already discussed, napthoquinones (vitamin K), pterins (folic acid) and the xanthones or flavones (vitamin P) might be the precursors or at least relatives, provided a nitrogen group is substituted in some position. Beyond this not much more can be said. It has been definitely determined that Cypridina luciferase will give no light when mixed with the vitamins mentioned above, and thus far it has not been possible to obtain light on mixing luciferase with compounds related to the vitamins or to known chemiluminescent substances like luminol, lucigenin, aesculin, lophin, etc., nor with a very large number of auto-oxidizable or easily oxidizable compounds. Likewise, no oxidizing enzyme system from nonluminous cells has yet been isolated that with certainty result in light emission on mixing with luciferin.

The discovery by Mason, (recently confirmed) of amino-acids in Cypridina luciferin, placing it among the natural peptids, like protamine or certain antibiotics (gramicidin and polymyxin), has given a new approach to the problem among the ostracods, a field now under intensive study in the Princeton laboratory. The yellow pigment of the chromopeptid is possibly the photogenic locus in the luciferin molecule. The yellow color may be a clue to structure, but a very tenuous one, as yellow pigments not necessarily recognized as playing an essential role in cell metabolism are legion in both the animal and plant kingdoms. As for the luciferins of other organisms, the collective evidence seems to point to the synthesis during evolution of quite different chemiluminescent substances in a number of light producing groups in the living world.

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# VISION

George Wald

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Light is a form of free energy. It makes two kinds of contribution to chemical processes: it supplies the energy of activation which excites molecules to react; and it provides the energy for endothermic reactions—the heat of reaction. In the first case it is, so to speak, only loaned to the reaction, which, if exothermic, may return the energy of activation manyfold. In the second case the light is used to do chemical work.

In the subjects of this symposium light appears in all its possible roles. In vision, it is used primarily to excite the retinal pigments. In photosynthesis, light supplies the energy to perform reductions. In bioluminescence, oxidations supply the energy emitted as light. I used to think that bioluminescence is vision in reverse; but it is more nearly the reverse of photosynthesis.

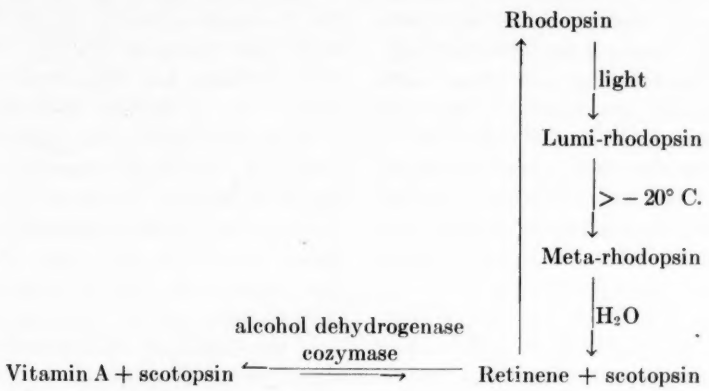
The retinas of most vertebrate eyes contain two kinds of light receptor: the rods, for vision in dim light, and the cones, for vision in bright light and color vision. Each of these organs contains a photosensitive pigment, which bleaches on exposure to light. Some aspect of this process leads to a nervous excitation which, transmitted from neurone to neurone along the optic pathways to the brain, ends in producing visual sensations.

Three such pigments are known: rhodopsin and porphyropsin in rods, and iodopsin in cones. All three pigments are carotenoid-proteins, proteins bearing carotenoid prosthetic groups to which they owe their color and sensitivity to light.

### I. RHODOPSIN

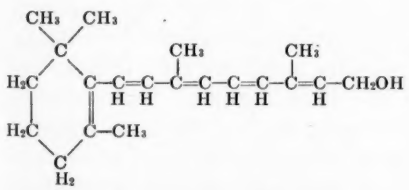
The red pigment rhodopsin was discovered in the rods of the frog retina by Franz Boll in 1876. It is characteristic of the rods of marine fishes

and land vertebrates (1). In the retina it takes part in the following complex of reactions (2, 3):

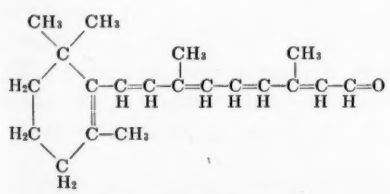



Rhodopsin, with $\lambda_{max}$ at 500 ± 2 mμ, is converted by light to the orange-red lumi-rhodopsin. At temperatures above −20°C this goes on to form meta-rhodopsin; and with access to water, meta-rhodopsin yields a mixture of the yellow carotenoid retinene and the colorless protein, scotopsin (4). (For reasons which will appear shortly, it seems advisable now to keep our original term, opsin, as the class name of the protein moieties of the visual pigments; and to distinguish within this class the proteins of rod vision, the scotopsins, from those of cone vision, the photopsins.)

Retinene is vitamin A aldehyde (5):



Vitamin A, $C_{19}H_{27}CH_2OH$



Retinene, $C_{19}H_{27}CHO$

In the retina or in solution, retinene is reduced to vitamin A by the enzyme alcohol dehydrogenase, working together with the coenzyme cozymase or DPN. The process involves only the transfer of 2 hydrogen atoms from reduced cozymase ($DPN—H_2$) to the aldehyde group of retinene, reducing it to the alcohol group of vitamin A (6–8):

$$C_{19}H_{27}CHO + DPN—H_2 \xrightleftharpoons{\text{alcohol dehydrogenase}} C_{19}H_{27}CH_2OH + DPN$$

retinene — vitamin A

The synthesis of rhodopsin from retinene and opsin is a spontaneous reaction. One has only to bring a mixture of these two substances in solution into the dark, to form rhodopsin. This is therefore, like all spontaneous reactions, an exergonic or energy-yielding process. It is the *bleaching* of rhodopsin that requires energy; and light, in addition to activating this molecule, is in part used also to bleach it (9).

The synthesis of rhodopsin from vitamin A and opsin is more difficult. The equilibrium between vitamin A and retinene lies far over toward the side of reduction—toward vitamin A. In the dark, however, scotopsin 'traps' retinene, removing it to form rhodopsin, and so displacing the equilibrium in the oxidative direction. The basic mechanism of rhodopsin synthesis, therefore, is the endergonic oxidation of vitamin A to retinene, coupled with the exergonic condensation of retinene and scotopsin to form rhodopsin. In the eye this process is aided by the influx of new vitamin A from the pigment epithelium, which obtains it from the blood circulation; by the provision of cozymase, the oxidant of vitamin A; and by respiratory enzymes, which keep cozymase oxidized. All these factors operate also *in vitro*, and in concert regenerate the visual pigment (10, 11).

From the diagram of the rhodopsin system

shown above, it would seem possible to reconstruct the entire system by mixing 4 substances in solution: vitamin A, scotopsin, alcohol dehydrogenase and cozymase. Such mixtures have been assembled, using vitamin A from fish liver oils, crystalline alcohol dehydrogenase from horse livers, and cozymase from yeast; only scotopsin needs to be obtained from the rods. Such a mixture, placed in the dark, forms rhodopsin. Taken out into the light, it bleaches; and replaced in the dark, it synthesizes more rhodopsin. This mixture of 4 substances performs all the reactions of the rhodopsin system (11).

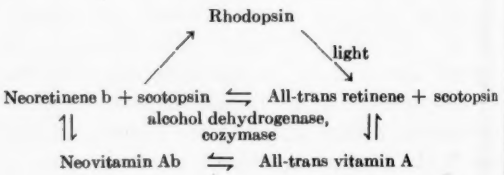
In making up this four-component system, however, not all vitamin A is effective. Rhodopsin is synthesized from liver oil vitamin A, but almost none from ordinary crystalline vitamin A or the commercial synthetic product. Like other carotenoids, vitamin A exists in a number of different molecular *shapes*, cis-trans isomers of one another (12, 13). The usual crystalline or synthetic vitamin A is primarily the all-trans isomer; this is ineffective in rhodopsin synthesis. Rhodopsin requires for its formation a cis isomer of vitamin A, present in liver oils (14, 15).

The alcohol dehydrogenase system is relatively indifferent to the cis-trans configuration of retinene or vitamin A. The dominant isomer-specific reaction in rhodopsin synthesis is the condensation of retinene with scotopsin. It is this that demands a specific cis isomer of retinene.

Five apparently cis-trans isomers of retinene have been isolated in crystalline condition: the all-trans isomer, originally prepared by Morton and his colleagues (5); neoretinenes *a* and *b*, first isolated in our laboratory; and isoretinenes *a* and *b*, first isolated at the Organic Research Laboratory of Distillation Products Industries in Rochester, N. Y. (16).

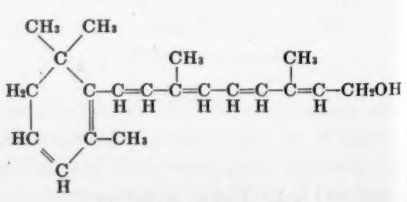
Neoretinene *b*, on incubation with scotopsin in the dark, yields rhodopsin, indistinguishable from that extracted from the dark adapted retina. Isoretinene *a* on similar treatment yields a light-sensitive pigment very much like rhodopsin, but with its $\lambda_{max}$ displaced to 487 m$\mu$; this is called iso-rhodopsin. The remaining isomers are inactive (15).

What isomer of retinene emerges when rhodopsin is bleached? This has proved to be the inactive, all-trans retinene. It must be isomerized to the active form, neoretinene *b*, before it can reenter the synthesis of rhodopsin. A cycle of carotenoid isomerization is therefore an intrinsic part of the rhodopsin system. The bleaching of rhodopsin yields all-trans retinene. This can be isomerized by simple exposure to light, constituting a second photochemical reaction in the rhodopsin system. In the eye, however, before this has gone very far, much of the all-trans retinene has been reduced to all-trans vitamin A. It appears that the eye tissues, perhaps principally the pigment epithelium, contain an enzyme, an isomerase, which catalyzes the isomerization of retinene and vitamin A. An important additional element, however, is the diffusion from the retina into the circulation of the all-trans vitamin A liberated in the bleaching of rhodopsin; and the recapture of neoretinene *b* from the circulation by the synthesis of rhodopsin. These exchanges with the blood keep the rhodopsin system in intimate contact with the metabolism and transport of vitamin A throughout the body, and with an external factor, its nutritional supply. With these transformations included, the rhodopsin system may be formulated as follows (15):

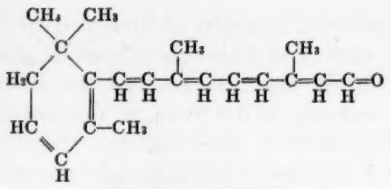



## II. PORPHYROPSIN

Porphyropsin is a purple light-sensitive pigment, associated characteristically with the rods of vertebrates which originate in fresh water—freshwater fishes, lampreys, and certain larval and adult amphibia (1). The $\lambda_{max}$ of porphyropsin in solution is at about 522 m$\mu$. The porphyropsin system is based upon different carotenoids from rhodopsin. Indeed it was the analysis of this visual system that led to the discovery of the new carotenoids later named vitamin $A_2$ and retinene$_2$ (17, 18). After many years of confusion, the structures of these substances have finally been established by total synthesis in the laboratory of E. R. H. Jones at Manchester (19).



Vitamin $A_2$, $C_{19}H_{23}CH_2OH$

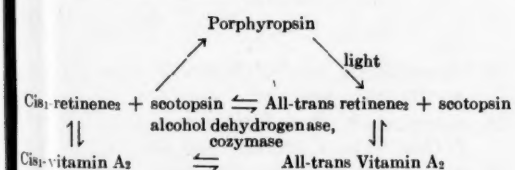


Retinene$_2$, $C_{19}H_{25}CHO$

The porphyropsin system shares most of its functional properties and some of its components with the rhodopsin system. Alcohol dehydrogenase and cozymase catalyze the same equilibrium between retinene$_2$ and vitamin $A_2$ as between retinene and vitamin A (7). The bleaching of porphyropsin yields what is apparently the all-trans isomer of retinene$_2$. This is inactive in resynthesizing porphyropsin. From a mixture of retinene$_2$ isomers, however, we have isolated 2 active fractions, which appear to contain 2 cis isomers of retinene$_2$, which can for the present be called cis$_1$ and cis$_2$. Neither has yet been completely purified, or crystallized. The cis$_1$ fraction, incubated in the dark with a solution of opsin from a freshwater fish, yields porphyropsin ($\lambda_{max}$ 523 m$\mu$). The cis$_2$ fraction, treated similarly, yields a comparable pigment with $\lambda_{max}$ 507 m$\mu$; this is iso-porphyropsin (20).

These fractions behave much the same with cattle as with freshwater fish opsin. It is true that the pigments obtained with cattle opsin are displaced toward shorter wavelengths than with fish opsin; cattle 'porphyropsin' has $\lambda_{max}$ 517 m$\mu$, and cattle 'iso-porphyropsin' $\lambda_{max}$ 501 m$\mu$. It should be recalled, however, that cattle rhodopsin is similarly displaced from bullfrog rhodopsin; the former has $\lambda_{max}$ 498 m$\mu$, the latter $\lambda_{max}$ 502 m$\mu$.

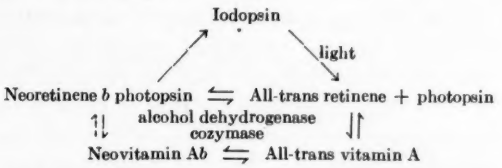
The opsins are so closely related in the rhodopsin and porphyropsin systems that it seems proper to regard them as belonging to the same family, the scotopsins. The rhodopsin and porphyropsin systems therefore share throughout the same proteins; it is only their carotenoids which differ. The porphyropsin system can be formulated as follows (20):




## III. IODOPSIN

Iodopsin is the only pigment of cone vision that has yet been identified. It was found in the chicken retina in 1937 (21). This retina contains a few rods among a large predominance of cones, and from it a mixture of iodopsin and rhodopsin was extracted.

Chicken iodopsin is a violet pigment, with $\lambda_{max}$ 562 m$\mu$. Recent experiments have shown that the carotenoids of the iodopsin system are identical with those of the rhodopsin system, even to cis-trans configuration. Only the opsins are different. With the opsins of cone vision designated as *photopsins*, the iodopsin system can be formulated as follows (22):




From the light-adapted chicken retina one can extract a colorless, carotenoid-free mixture of the proteins of rod and cone vision, scotopsin and photopsin. On incubating this in the dark with neoretinene *b*, one obtains a mixture of rhodopsin and iodopsin, indistinguishable from that extracted from the dark adapted chicken retina.

Table 1. Composition of visual photopigments and of their cis-trans artifacts, the iso-pigments

| Pigment | Protein (Opsin) | Carotenoid (Retinene) | Absorption maximum m$\mu$ |
|---|---|---|---|
| Rhodopsin | Scotopsin | Neoretinene *b* | 500 |
| Porphyropsin | Scotopsin | Cis$_1$-retinene$_2$ | 522 |
| Iodopsin | Photopsin | Neoretinene *b* | 562 |
| Iso-rhodopsin | Scotopsin | Isoretinene *a* | 487 |
| Iso-porphyropsin | Scotopsin | Cis$_2$-retinene$_2$ | 507 |
| Iso-iodopsin | Photopsin | Isoretinene *a* | 515 |

Just as isoretinene *a* yields iso-rhodopsin when incubated with scotopsin, it yields a similarly displaced pigment, iso-iodopsin, on incubation with photopsin. The $\lambda_{max}$ of iso-iodopsin is at about 515 m$\mu$.

## IV. RECAPITULATION

The composition of the light-sensitive pigments described above is summarized in table 1. It should be noted that the iso-pigments must, at least at present, be regarded as artifacts. The retinas which we have examined contain only rhodopsin, porphyropsin and iodopsin. Either the isoretinenes are not formed in the animal body, or some mechanism excludes them from participation in the visual processes.

### V. PHYSIOLOGICAL CORRELATIONS

A number of basic visual phenomena have their source in the chemical and physical properties of the visual pigments.

The spectral sensitivity of rod vision, after it has been corrected for the distortions imposed upon it by the transmission characteristics of the ocular media, is virtually identical with the absorption spectrum of rhodopsin. The same relationship exists also between the spectral sensitivity of cone vision and the absorption spectrum of iodopsin. It has long been known that as the eye goes from dim to bright light, from rod to cone vision, its sensitivity shifts toward the red. This is the Purkinje phenomenon. It involves no more than the shift of vision from dependence upon rhodopsin ($\lambda_{max}$ 500 m$\mu$) in dim light to iodopsin ($\lambda_{max}$ 562 m$\mu$) in bright light.

For many years we have believed that the process of dark adaptation reflects physiologically the synthesis in the rods and cones of the photosensitive pigments bleached by a previous exposure to light. In the human eye, the cones dark-adapt very quickly compared with the rods; cone dark adaptation is complete within about 5 minutes, while rod dark adaptation occupies about an hour (23). The synthesis of iodopsin and rhodopsin in solution exhibits similar time relations. At room temperature, the synthesis of chicken iodopsin is completed within 3–5 minutes, while that of rhodopsin takes an hour.

With cone vision one confronts the problem of color vision. In the chicken, as in certain other birds and in turtles, color vision requires no more than a single cone photosensitive pigment such as iodopsin, for in these animals the cones are equipped with a system of minute color filters, one in each cone. These are colored oil globules, lying at the junctures between the inner and outer segments of the cones, so that they mask the light-sensitive outer segment. There are 3 colors of globule—red, orange and light yellow—and also colorless globules. The filter pigments of the chicken retina are all carotenoids: the red astaxanthin, a golden mixture of xanthophylls, and a light yellow carotene (24). To these an almost colorless carotenoid, galloxanthin, has recently been added, which may act principally to exclude the ultraviolet (25). In combination with the single photopigment iodopsin, these filters could provide the basis for a tetrachromatic system of color vision, just as a similar arrangement of micro-color filters, in combination with the single photosensitive substance silver bromide, was used in the old autochrome process of color photography.

The mammalian retina lacks such color filters, and here it is thought that color vision is probably mediated by a number of different light-sensitive pigments, distributed among the cones. The visual systems reviewed above reveal three ways in which such a variety of photosensitive pigments might be achieved. A single protein might combine with different carotenoids, as in rhodopsin and porphyropsin; or different proteins might couple with the same carotenoid, as in rhodopsin and iodopsin; or different cis-trans isomers of the same carotenoid might combine with the same protein, as in rhodopsin and iso-rhodopsin. If as yet we know almost nothing of the mechanism of mammalian color vision, it is not for lack of biochemical possibilities.

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# PHOTOSYNTHESIS: A THERMODYNAMIC PERFECTION OF NATURE

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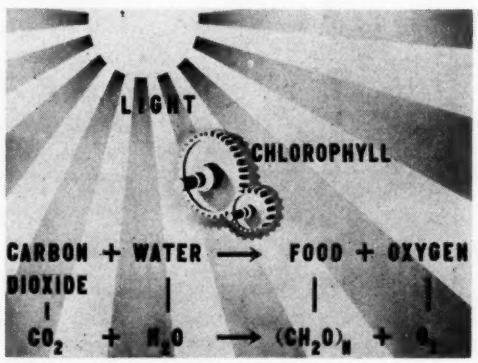
*The changing of Light into Bodies is very conformable to the Course of Nature.*

SIR ISAAC NEWTON. Knt. *Optiks*, Query 30 (1718)

THE green leaf is one of the world's most active chemical laboratories, as well as one of the most mysterious. Photosynthesis is the chief business carried on in the tiny laboratory of the chloroplast. In photosynthesis, the plant captures energy from the sunlight, carbon dioxide from the air, and water from the soil. From these very basic ingredients are formed, as indicated in slide 1, the food—carbohydrate, protein, and fat—without which man and all other animals would starve, and the oxygen gas without which we would all suffocate. This production of organic matter and ameliorated air takes place when geared to the absorption and utilization of light by means of the colored photocatalyst chlorophyll which, with or without the aid of other accessory plant pigments, can make use of virtually all of the visible spectrum, and at times the near ultra-violet and near infra-red as well.

The inner secrets of the magic-like ability of plants to change light energy into chemical energy, in a manner so conformable to the Course of Nature, are still highly elusive, and constitute one of the great challenges to biochemists today. On the other hand, in the last few years Otto Warburg and collaborators (1–19) have succeeded in obtaining the answer to the most fundamental question that may be asked concerning the over-century-old, photosynthetic information presented in slide 1.

The basic question, as to *how much*, or what fraction, of the light energy absorbed by the green plant can be converted into chemical energy under the most favorable conditions, can now be answered with experimental, even if not semantic[1] certainty. A presentation of important factual information bearing on this question will be the main theme of this paper, which obviously cannot, in the time at hand, encompass many other recent developments in the field of photosynthesis, prominent among which are the detailed chemical paths of the transformation of inorganic to organic carbon (being carried out so effectively with the aid of isotopic tracers), partial photosynthetic reactions obtained with broken up cell material (chloroplasts, grana, chromo-




Slide 1. BASIC equation for steady state photosynthesis.

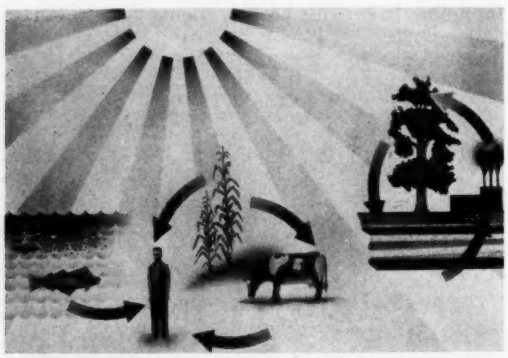
[1] Every scientific age has, of course, its sophists, and the mid-Twentieth Century has produced a rare variety known as photosophist, which specializes not in counting equine molars or in abhorring vacua, but in doubting whether oxygen is really oxygen or its photosynthetic production really photosynthesis.

protein, isolated enzymes), chlorophyll structure and spectra, fluorescence and luminescence studies, the limiting dark Blackman reaction, new types of photosynthetic organisms, 'algae farming', and non-protoplasmic photosynthesis.

Why is the question of the efficiency of photosynthetic energy transformation so fundamentally important?

The atom bomb has of late become a symbol of the ultimate in sources of power. Yet, even the

Slide 2. COMPARISON of incident solar energy with energy derived from Hiroshima atom bomb.



Slide 3. TRANSFORMATIONS of organic matter produced by various photosynthetic organisms.

presently foreseeable potential of atomic energy is dwarfed by the prodigious quantities of energy showered onto the earth each day by the sun. As indicated in slide 2, the total solar energy falling on New York City in one day alone is equivalent to the energy released in some 240 Hiroshima atom bombs; and the total energy falling daily on the continental United States is estimated to be several thousand times the amount of energy used by the contained population in one day for food, heating, lighting, transportation, manufacturing, etc.

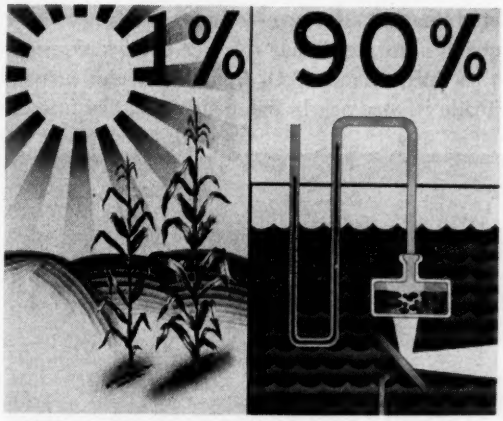
Unfortunately, not all of the energy beamed onto the earth by the sun is utilizable by man. Only that portion which is absorbed and effectively transformed by green plants may become useful, in so far as only green plants are known to have the capacity for absorbing and storing the sun's energy in notable degree. Since man and animals live by drawing from this reservoir a steady supply of the energy-rich compounds which they use as food, they are in effect parasites on the plants. Man in particular is dependent upon the green plants, for he derives from them not only food, and the fibers from which he makes his clothing, but all present-day sources of industrial power as well. The coal, oil, and natural gas which we burn to produce heat, or to produce steam, or to produce electrical power, are in reality the decomposition products of the trees and plants of past ages. Thus, the coal fire in one's furnace is in reality a tiny portion of the sun's inferno captured by green plants eons ago and stored in the form of potential energy.

Now, to have supplied virtually all of the food, fiber, and energy for life on a planet is a big job, and when one considers that the green plants have performed this staggering task within their delicate tissues, it is indeed a marvel! Nevertheless, one may ask whether the green plants, which have had ages to accomplish this, are effective enough to use with sufficient speed and efficiency the solar energy arriving in this and future ages in such manner as to provide an important, even if not total, substitute for past stores, and thereby reduce the drain on our accumulated resources. In this connection it is well to bear in mind that, as indicated in slide 3, regardless of the photosynthetic efficiency, whether very high or very low, with which organic matter might be produced from inorganic carbon dioxide by means of cultivated crops, forests, grasslands, or aquatic microorganisms, the further transformations of this organic matter from one plant organism into another, from plant into animal, or animal into animal, involve large progressive losses of organic matter, of the order of 90% at each stage. Thus, to make 1 pound of very little fish takes 10 pounds of algal food consumed; to make 1 pound of little fish that eat very little fish takes 10 pounds of the latter; and to make 1 pound of a big fish takes 10 pounds of little fish; so that to make 1 pound of a very big fish in such a cycle may require on the order of 1,000,000 pounds of algae; and, similarly, a further 10-fold loss would

be incurred when the very big fish is eaten and utilized by a rapidly growing child.

If, perchance, the photosynthetic efficiency of the total global flora, regarded as a machine, is inadequate to meet the foregoing extended food and related energy needs of the rest of the globe, to what extent can this be improved by man and science?

The answer to this question may be anticipated and epitomized at this point by means of slide 4, which indicates diagrammatically that whereas in Nature a photosynthetic efficiency of 1% for the conversion of light energy into chemical energy represents nearly an upper limit, nevertheless, in the laboratory it is now readily possible to obtain an efficiency of at least 90% under optimum experimental conditions, employing green algae in a small vessel and measuring upon illumination their oxygen production by means of a manometer attached to the vessel.




Slide 4. COMPARISON of photosynthetic efficiencies in the field and in the laboratory.

SLIDE 5. IMPORTANCE OF $O_2$ GAS AS A MEASURE OF PHOTOSYNTHETIC ENERGY EXCHANGE IN THE STEADY STATE

| PROCESS | EQUATION | CALORIES ($\Delta H$) | $\gamma = CO_2/O_2$ | |
|---|---|---|---|---|
| Respiration | $O_2 + 1/6\ C_6H_{12}O_6 = H_2O + CO_2$ | +112,000 | −1.00 | (6) |
| Photosynthesis | $CO_2 + H_2O = 1/6\ C_6H_{12}O_6 + O_2$ | −112,000 | −1.00 | (1) |
| | $6/7\ CO_2 + 8/7\ H_2O = 2/7\ CH_2OHCHOHCH_2OH + O_2$ | −114,000 | −.85 | (2) |
| | $4/3\ CO_2 + H_2O = 1/3\ COOHCHOHCH_2COOH + O_2$ | −106,000 | −1.35 | (3) |
| | $9/13\ CO_2 + 9/13\ H_2O = 1/26\ C_{18}H_{36}O_2 + O_2$ | −105,000 | −.69 | (4) |
| | $4\ CO_2 + 2\ H_2O = 2\ COOHCOOH + O_2$ | −120,000 | −4.00 | (5) |

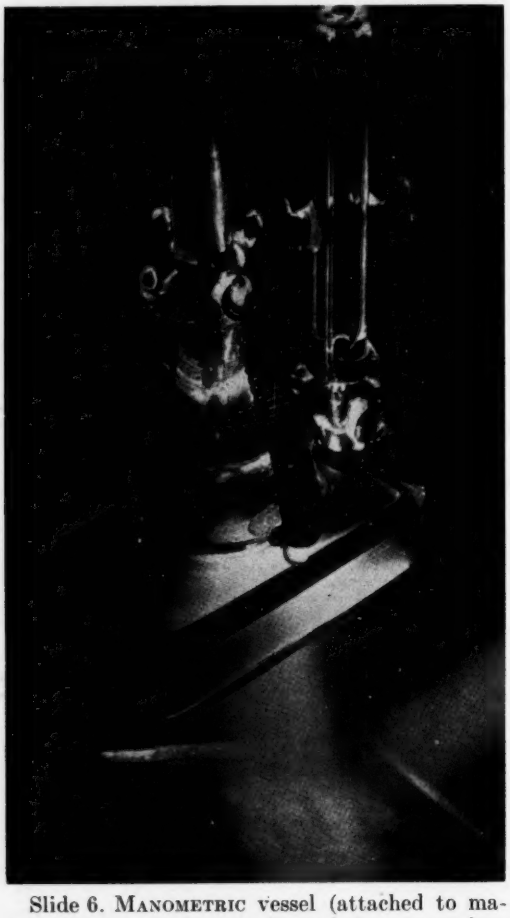
Required Energy/Mol $O_2$: 112,000 Cal. ~ 3 Mol Quanta of Red Light ~ 2 Mol Quanta of Blue Light

As to future predictions bearing on the question last posed, we may content ourselves at this point with recalling that when James B. Conant, retired President of Harvard, gave his 'crystal ball' address before the American Chemical Society two years ago, he stated that, in this ball, he saw solar energy as the dominating factor in the production of industrial power by the year 2000. A few months later, Chairman Gordon Dean of the Atomic Energy Commission spoke of the solution to photosynthesis as a means by which man might "create virtually unlimited supplies of both food and fuel" and win "his independence from the natural resources now so rapidly being depleted." Of 3 possible solutions to our vanishing supplies of energy, Chairman Dean noted that one, nuclear energy, had been partly achieved in the atomic bomb, and the other 2 solutions depend on photosynthesis: by duplicating Nature's work and increasing her efficiency we might greatly expand our food supply, or, more important, by mastering a detailed understanding of photosynthesis we might harness the sun for power.

## MAXIMUM PHOTOSYNTHETIC EFFICIENCY

**$O_2$ Production as Criterion of Energy Storage.** The production of oxygen gas is the most important, unambiguous, and constant measure of light energy transformation in steady state photosynthesis. As indicated in slide 5, about 112,000 (±8000) calories of heat energy are required to produce a mole of $O_2$ from $CO_2$ and $H_2O$, regardless of whether the organic product simultaneously formed is carbohydrate, or much more highly reduced material such as glycerol or fatty acid, or much more oxidized material such as oxalic acid; and regardless of whether the ratio '$\gamma$ = $CO_2$ consumed/$O_2$ produced' is near unity or quite far from it (−0.69 to −4.0). The relationship '$O_2$ produced/calories required' is clearly far more constant than such ratios as '$CO_2$ consumed/calories required', '$\gamma$/calories required', or the nature of the organic material formed in relation to calories required, all pro-

viding, of course, that the initial reactants are exclusively $CO_2$ and $H_2O$ properly balanced stoichiometrically. Another method of indicating the preferred status of $O_2$ measurements for the determination of the efficiency of energy conversion is to recall that the free energy required to produce a mole of $O_2$ from water and carbon dioxide is very nearly independent of the fate of



Slide 6. MANOMETRIC vessel (attached to manometer by steel spring) used for measuring, photosynthetically or actinometrically, quanta of light received from a collimated beam.

the hydrogen atoms involved, and is approximately 115,000 calories (20), as in

$$2CO_2 + 2H_2O \rightarrow 2HCOO^- (aq) + 2H^+ (\text{pH } 7) + O_2, \text{ or}$$

$$\tfrac{2}{3}CO_2 + \tfrac{4}{3}H_2O \rightarrow \tfrac{2}{3}CH_3OH (aq) + O_2.$$

The relationships might well be quite otherwise if the above equations and *equations 1* to *5* in slide 5 contained initial reactants other than $CO_2$ and $H_2O$. Thus, for the production of one mole of $O_2$ from $CO_2$ and $H_2O_2$, the heat of reaction would be not 112,000 but 59,000 calories required, and for the production of 1 mole of $O_2$ and 2 moles of cobaltodihistidine from 1 mole of the chelate peroxide oxy-bis(cobaltodihistidine), the heat of reaction (21) would be 38,000 calories required (standard free energy = 9000 calories required/mole), and the free energy ordinarily close to zero. Whereas in steady state photosynthesis the initial reactants ordinarily are $CO_2$ and $H_2O$, this need not be the case at all during transition phases of photosynthetic mechanism where intermediates or catalysts may be involved in a stoichiometric sense.

The view is sometimes advanced that, in long time experiments, production of organic matter might be a better photosynthetic measure than $O_2$ production, especially if the heat of combustion of the total formed organic product were determined, in lieu of the virtually impossible identification and analysis of each and every organic compound produced. However, in any experiments carried out long enough to yield sufficient accumulation of organic matter to permit its satisfactory measurement, it is likely that the conditions would be such as to preclude obtaining *maximum* percentage efficiency of energy transformation; in particular, the light intensities involved would probably be too high. Although determinations of organic matter formation in photosynthesis are relatively insensitive, nevertheless they can be very useful and definitive in connection with photosynthetic yields under *sub-maximal* conditions.

Clearly, the production of a mole of oxygen in efficient steady state photosynthesis may be regarded as the most certain measure of the transformation of some 110,000–115,000 calories of light energy into chemical energy, stored in the form of the equivalent organic matter produced regardless of whether its chemical composition is known or unknown. In slide 6 is illustrated an actual manometric vessel (rather than the diagrammatic vessel, slide 4) that can be readily used to measure oxygen production and photosynthetic efficiency.

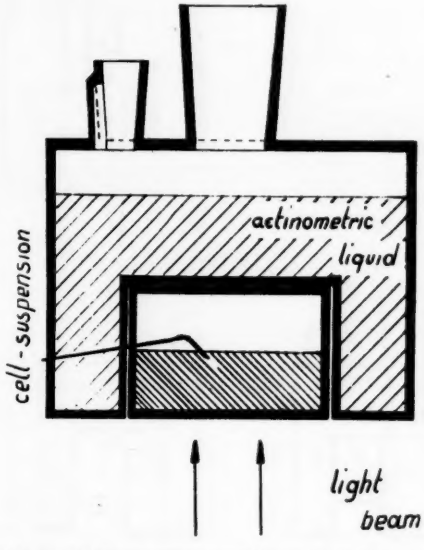
**Light Measurements in Manometric Vessels.** The vessel shown in slide 6 epitomizes the relative simplicity of making photosynthetic efficiency measurements, if one wishes to make them simple. In addition to determining the $O_2$ produced from pressure changes that can be read on the manometer attached to the vessel, it is only

further necessary to measure the amount of light absorbed by the algal contents of the vessel from the collimated beam, in order to determine the efficiency ratio '$O_2$ produced/light absorbed'. Two general situations may arise.

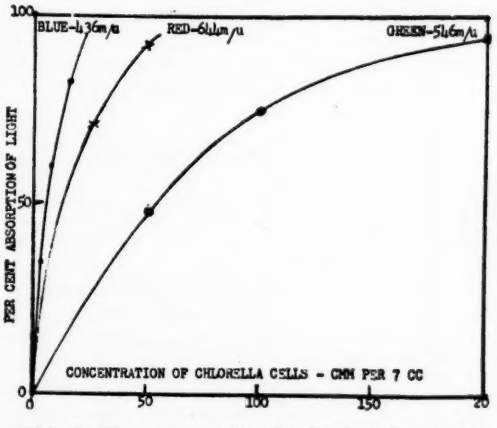
The simplest situation is where the algal suspension is so dense that it absorbs all of the light, in which case one may fill a vessel of the type shown in slide 6 with actinometric fluid instead of algal cells and, at low light intensities, observe an *absorption* of one molecule of oxygen for each quantum of light absorbed (22), in direct accordance with Einstein's law of photochemical equivalence, according to which chemical substances affected by light react in a primary process through the exact equality of 1 molecule altered per 1 quantum of minimum energy content absorbed.

In the more general case of partial absorption of light by an algal suspension, it is necessary to employ a slightly more elaborate type of vessel, as illustrated in slide 7 (5, 8, 12), in which actinometric fluid is contained in the larger vessel adequately surrounding an alga-containing vessel equivalent in dimensions to that shown in slide 6. At low light intensities, the manometrically measurable oxygen consumed by the actinometric fluid in the larger vessel is a quantitative measure, molecule of oxygen consumed per quantum absorbed, of the light transmitted by the alga-containing vessel. The percentage of light transmitted will vary from 100–0% (or light absorbed from 0–100%) as the concentration of algae in the inner vessel is progressively increased, as in slide 8, which shows curves obtained with different wave lengths of light for a given suspension of Chlorella cells varied in concentration from 0–200 cmm of moist cells per 7 cc of medium (8). The arrangement in slide 7 does not permit of strictly simultaneous photosynthetic and actinometric measurements, since the alga-containing vessel is not connected with a manometer. The more complex vessel illustrated in slide 9 provides a method for measuring both the light absorption of, and photosynthesis by, a given cell suspension (6). In the foregoing methodology it may become necessary to take due account of the fact, illustrated in slide 10, that as the light intensity is increased the actinometric yield ($O_2$ consumed/quanta absorbed) may fall to a level somewhat below unity (8). Another purely physical method for measuring light transmission by an algal suspension, alternative to the manometric procedure of slide 7, can be accomplished by means of a suitable integrating sphere, to be referred to in a later slide.

**Minimum Quantum Requirement of Photosynthesis.** As indicated in slide 5, some 112,000 calories are required for the production of 1 mole



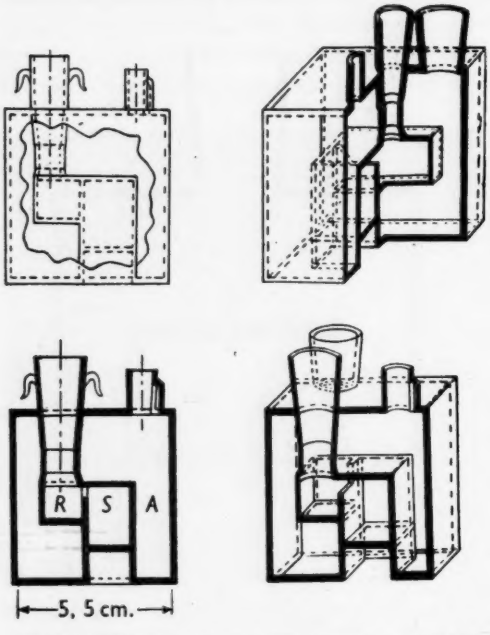

Slide 7. MANOMETRIC vessel used for measuring percentage light absorption (transmission) by a given algal suspension (5, 8, 12).



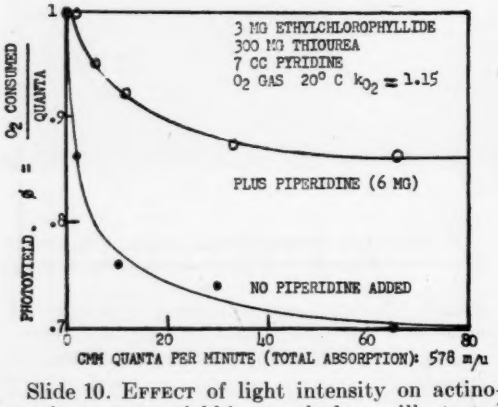

Slide 8. TYPICAL results obtained with vessel of type in slide 7 for absorption of light of different wavelengths by increasingly dense suspensions of algae cells (8).

of oxygen from carbon dioxide and water in steady state photosynthesis and with exclusion of transition stages of mechanism. The energy of 1 mole quantum of red light is of the order of 41,000 calories at the effective long wavelength limit. Therefore, on thermodynamic

grounds, overall steady state photosynthesis with red light cannot be a simple 1-quantum reaction, but must involve a minimum of 2.7 (112,000/41,000) quanta per molecule of $O_2$.

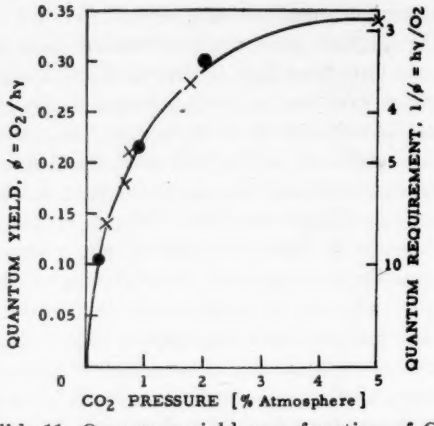



Slide 9. COMPOUND two-manometer vessel for measuring actinometrically in *A* the light transmitted from *S*, containing cells whose metabolism is measured by manometer above the reagent compartment *R*. Similarly useful for measuring fluorescence transmitted into *A* by a substance in *S*. Incident light enters *S* from bottom (6).

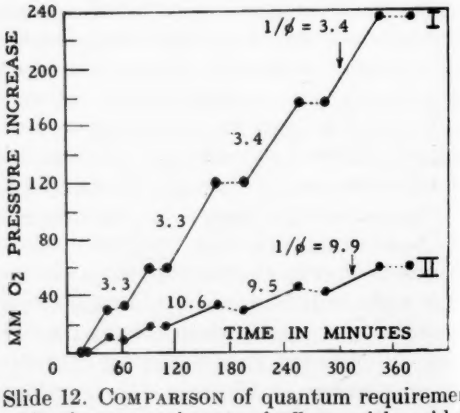

3 MG ETHYLCHLOROPHYLLIDE
300 MG THIOUREA
7 CC PYRIDINE
$O_2$ GAS 20° C $k_{O_2} = 1.15$
PLUS PIPERIDINE (6 MG)
NO PIPERIDINE ADDED
PHOTOYIELD, $\phi$ = $O_2$ CONSUMED / QUANTA
CMM QUANTA PER MINUTE (TOTAL ABSORPTION): 578 mμ

Slide 10. EFFECT of light intensity on actinometric quantum yield in vessel of type illustrated in slide 6 (8).

A large amount of experimental work has shown that in steady state photosynthesis it is possible to approximate this value experimentally fairly closely if sufficient care is taken to maintain optimum conditions. Among the most important conditions are: adequate $CO_2$ pressure, on the order of 2–5% atmosphere (slides 11 and 12




Slide 11. QUANTUM yield as a function of $CO_2$ pressure (13, 18). (●) Measurements made by the 1-vessel method in bicarbonate-carbonate solution at 20°C. (×) Measurements made by the 2-vessel method in acid medium.

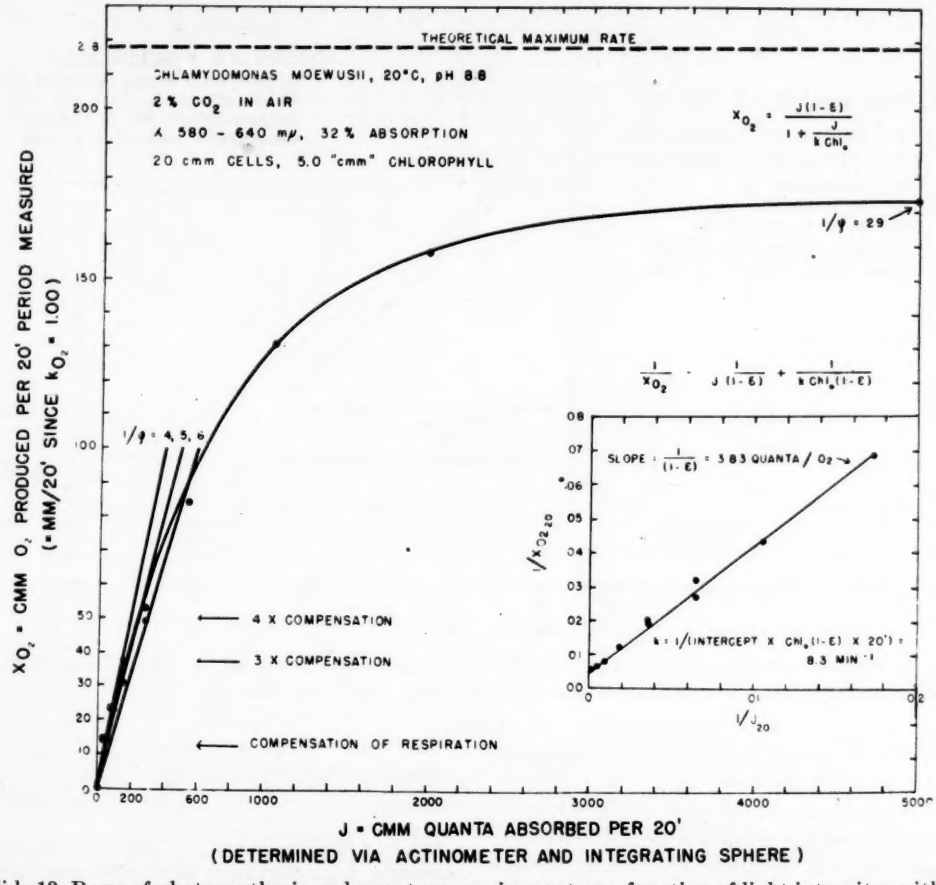



Slide 12. COMPARISON of quantum requirement in bicarbonate-carbonate buffers with widely different $CO_2$ pressures (18). *Curve I:* 2% $CO_2$, average quantum requirement 3.35. *Curve II:* 0.22% $CO_2$, average quantum requirement 10. Single vessels of equal dimensions employed for each curve (vol. = 16.6 cc, vol. of medium 7.0 cc, $k_{O_2}$, 20° = 0.915), with equal illumination (blue light, wavelength 436 mμ, 3.3 cmm of quanta/min, totally absorbed). The respiration was compensated by white light from a Hg lamp. The dotted curve parts approximately parallel to the x-axis: white without blue; the solid curve parts: white plus blue. The blue light, when provided, was continuous, not intermittent.

A large amount of experimental work has shown that in steady state photosynthesis it is possible to approximate this value experimentally fairly closely if sufficient care is taken to maintain

with *Chlorella pyrenoidosa*) and sufficiently low light intensity, of the order of less than 1 quantum of light absorbed/molecule of chlorophyll/minute (slide 13 with *Chlamydomonas moewusii*).

The experiment of slide 13 was carried out under virtually ideal conditions from all points of view. At any given incident light intensity, all algal cells were virtually equally illuminated, of the vessel at all times. Under such ideal conditions, it is possible to obtain not only a truly hyperbolic curve, but high photosynthetic yields (quantum requirements of only 3 to 5 per $O_2$

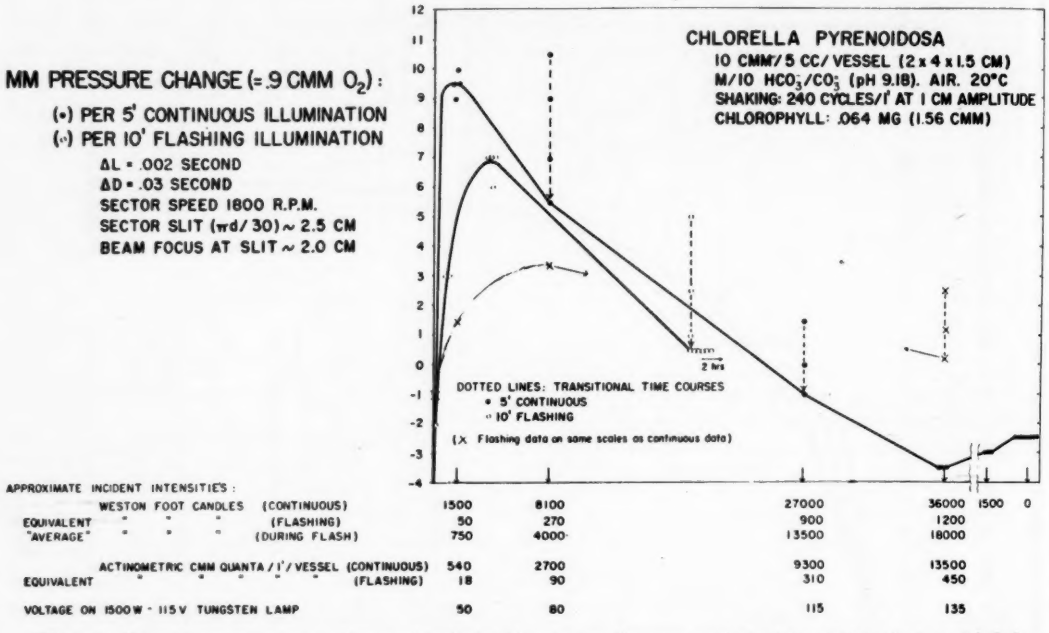

THEORETICAL MAXIMUM RATE
CHLAMYDOMONAS MOEWUSII, 20°C, pH 8.8
2% CO2 IN AIR
λ 580 - 640 mμ, 32% ABSORPTION
20 cmm CELLS, 5.0 "cmm" CHLOROPHYLL
1/φ = 29
1/φ = 4, 5, 6
4 X COMPENSATION
3 X COMPENSATION
COMPENSATION OF RESPIRATION
SLOPE = 3.83 QUANTA / O2
k = 1/(INTERCEPT X Chl.(1-ε) X 20') = 8.3 MIN⁻¹
XO2 = CMM O2 PRODUCED PER 20' PERIOD MEASURED (= MM/20' SINCE kO2 = 1.00)
J = CMM QUANTA ABSORBED PER 20'
(DETERMINED VIA ACTINOMETER AND INTEGRATING SPHERE)

Slide 13. RATE of photosynthesis and quantum requirement as a function of light intensity, with thin algal suspension contained in vessel of type shown in slide 6, but shaking in a single uniform light beam that covered exposed bottom area of vessel at all times. Quantum requirement of 3.5–5.0/$O_2$ produced up to several times compensation of respiration (12 ± 1 cmm $O_2$ consumed for 20′). Four day old culture of *Chlamydomonas moewusii* grown at pH 6.7 to 300 cmm/100 cc, under sterile conditions (Feb. 25, 1952), centrifuged and taken up in M/5 $NaHCO_3$–$Na_2CO_3$ pH 8.79. 7.7 cc buffer medium containing 20 cmm moist cells of 1.05% chlorophyll (= 0.21 mg = 5.0 cmm chlorophyll) added to 18.2 cc vessel, of bottom area 3 × 5 cm², shaken in uniform light beam 6 × 8 cm². Percentage absorption measured in modified Ulbricht integrating sphere with Photovolt recording instrument. All readings for 20′ periods taken randomly (thus, proceeding along curve downward, points shown taken at periods 16, 15, 14, 7, 6, 13, 2, 5, 3, 10), with points for respiration (for J = O, dark) at periods 1, 4, 8, 9, 11, and 18. Duration of experiment ca. 6 hours. Inset shows closely hyperbolic nature of the yield-intensity function, since reciprocal of yield plotted against reciprocal of intensity gives (within experimental error) a straight line, whose slope over the entire range indicates a limiting quantum requirement of 3.8/$O_2$ produced for light effectively absorbed, and whose extrapolated intercept at 1/J = O indicates at high light intensities a limiting dark reaction whose k value is 8.3 reciprocal minutes.

since a thin suspension transmitting over two-thirds of the incident light was employed, and the vessel was shaken in a single large uniform beam of light that covered the entire bottom area

produced) up to several times the compensation point of respiration, as shown in the slide. The experiment in this slide answers succinctly all significant experimental objections that have

hitherto been raised to the view that no more than 3 to 4 quanta of light need be required per molecule of oxygen gas produced in long-term, steady-state photosynthesis under optimum conditions of illumination and carbon dioxide pressure. Many of the procedural improvements in the type of experimentation shown in this slide were introduced as a result of such objections.


MM PRESSURE CHANGE (=.9 CMM $O_2$):
(•) PER 5' CONTINUOUS ILLUMINATION
(◦) PER 10' FLASHING ILLUMINATION
ΔL = .002 SECOND
ΔD = .03 SECOND
SECTOR SPEED 1800 R.P.M.
SECTOR SLIT (πd/30) ~ 2.5 CM
BEAM FOCUS AT SLIT ~ 2.0 CM
CHLORELLA PYRENOIDOSA
10 CMM/5 CC/ VESSEL (2 x 4 x 1.5 CM)
M/10 $HCO_3^-/CO_3^=$ (pH 9.18). AIR. 20°C
SHAKING: 240 CYCLES/1' AT 1 CM AMPLITUDE
CHLOROPHYLL: .064 MG (1.56 CMM)
2 hrs
DOTTED LINES: TRANSITIONAL TIME COURSES
• 5' CONTINUOUS
◦ 10' FLASHING
(X Flashing data on same scales as continuous data)
APPROXIMATE INCIDENT INTENSITIES:
WESTON FOOT CANDLES (CONTINUOUS) 1500 8100 27000 36000 1500 0
EQUIVALENT " " " (FLASHING) 50 270 900 1200
"AVERAGE" " " " (DURING FLASH) 750 4000 13500 18000
ACTINOMETRIC CMM QUANTA / 1' / VESSEL (CONTINUOUS) 540 2700 9300 13500
EQUIVALENT " " " " " (FLASHING) 18 90 310 450
VOLTAGE ON 1500W - 115V TUNGSTEN LAMP 50 80 115 135

Slide 14. PHOTOSYNTHETIC maxima and solarization in continuous and flashing light of very high intensity, in uniform beam larger than, and always covering, vessel bottom.

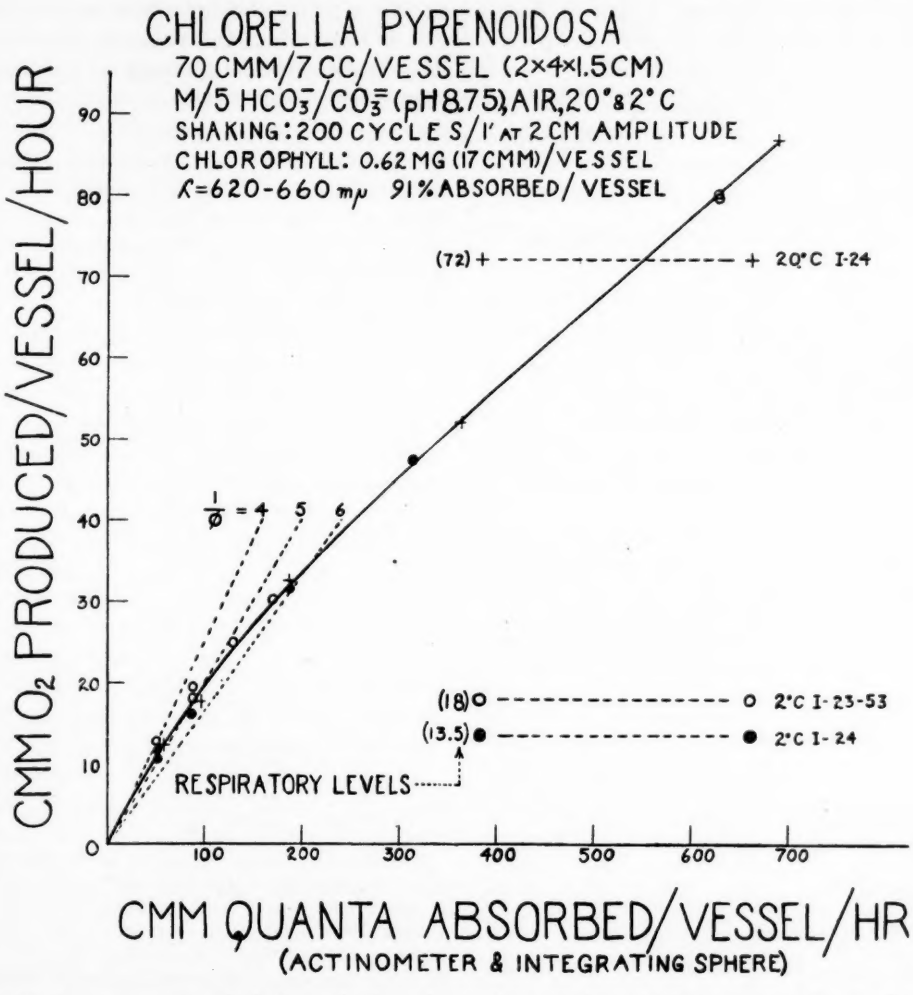
As light intensity is progressively increased, the quantum yield progressively falls (quantum requirement progressively increases), and the rate of photosynthesis increases to, and passes through, a maximum,[2] as indicated in slide 14, and then progressively decreases to negative values that may at times attain nearly half the maximum rate of photosynthesis but of reverse sign ($O_2$ consumption in light, instead of $O_2$ production).

The rate of photosynthesis and quantum requirement ($1/\phi$) at low light intensity are, as indicated in slide 15, totally independent of

[2] Photosynthesis as a function of high light intensity passes through an optimum not only in continuous illumination (23), but, as now shown here for the first time (slide 14), in flashing light also. Both continuous and flashing photosynthesis-intensity functions show optima at ~5000 foot candles white light (depending considerably upon previous culture adaptation), and virtual elimination of positive photosynthesis by ~25,000 f.c. Such solarization curves (reduced photosynthesis near and beyond the optima) are fairly reversible, but require measureable periods of time (minutes) for back and forth reversal from one curve point to another; marked and progressive irreversibility occurs only at light intensities high enough to effect net oxygen consumption ('negative photosynthesis' or photooxidation). In flashing light, reversible solarization (like Weston photometer response) depends mainly upon intrinsic ('instantaneous') rather than time-integrated intensity, whereas the converse is more nearly true of photosynthesis proper (and bolometer response). The apparent approach to photosynthetic flash saturation reported by, e.g., EMERSON AND ARNOLD (24), WELLER AND FRANCK (25), and EHRMANTRAUT AND RABINOWITCH (26), results largely from onset of solarization even before the optima are reached. In such experiments, the added phenomenon of solarization makes attainment of true flash saturation impossible, and even unapproachable. Hence, the concepts derived from such experiments, of a Blackman reaction dark time of ~0.01 sec, of a chlorophyll unit of ~200 to 2000 molecules, or of a catalyst B, are rendered superfluous by direct experimental evidence independent of, and in addition to, that reported previously (16); as Weller and Franck state (25, p. 1364): "it would be futile to draw any conclusions about the duration of the dark reactions while using light flashes whose intensity is not great enough to produce flash saturation."

temperatures between 2° and 20°C, in spite of a 4- to 5-fold difference in rate of respiration at these two temperatures. Not only is photosynthesis at low light intensities virtually independent of respiration over a wide range, but, conversely, as indicated in slide 16, low light intensity has very little if any measurable effect on respiration. At low light intensities, photosynthesis and respiration are, or (as in slides 15 and 16) may be made to be, quantitatively independent of each other.

In slides 11–13 and 15, limiting low quantum requirements of the order of 3–4 (maximum quantum efficiencies of 90–70%) have been described with respect to $O_2$ production, at given, constantly maintained pressures of carbon dioxide. Slide 17 provides data showing the same type of low quantum requirement result obtained


CHLORELLA PYRENOIDOSA
70 CMM/7 CC/VESSEL (2×4×1.5 CM)
M/5 HCO3⁻/CO3⁼ (pH 8.75), AIR, 20° & 2° C
SHAKING: 200 CYCLES/1' AT 2 CM AMPLITUDE
CHLOROPHYLL: 0.62 MG (17 CMM)/VESSEL
λ=620-660 mμ 91% ABSORBED/VESSEL
(72) + + 20°C I-24
1/Φ = 4 5 6
(18) o o 2°C I-23-53
(13.5) ● ● 2°C I-24
RESPIRATORY LEVELS
CMM O2 PRODUCED/VESSEL/HOUR
0 10 20 30 40 50 60 70 80 90
100 200 300 400 500 600 700
CMM QUANTA ABSORBED/VESSEL/HR
(ACTINOMETER & INTEGRATING SPHERE)

Slide 15. INDEPENDENCE of photosynthesis and quantum requirement upon temperature and rate of respiration. Beam of light, striking bottom of vessel, ca. 1.8 cm in diameter. All photosynthesis measurements made for 20′ except lowest point, 600′; third lowest, 120′; fourth lowest, 100′; and 40′ for the 2nd, 8th, and 10th points; 15′ for the 12th and 15th points and 10′ for the 7th point; all taken over a continuous run lasting two days. Proceeding up the curve, the period order of taking the points was 12, 18, 23, 5, 17, 13, 10, 22, 3, 11, 4, 16, 19, 15, 21, 6, 8, and 20, with missing periods employed for measurements of respiration (0 light intensity). Respiration measurements were also made in another identically prepared adjacent vessel wrapped in black cloth which gave respiratory results strictly identical with those of the main vessel; this situation is easy to obtain essentially indefinitely at 2°C, but at 20°C for usually not more than a few hours.

SLIDE 16. DOES RED LIGHT OF LOW INTENSITY ACT ON RESPIRATION?

300 cmm Chlorella cells in 7 cc culture medium, pH 5.0, in vessel with 2 sidearms each containing 0.3 cc 1 N NaOH and glass beads. (Readings started after 40 minutes shaking for equilibration.) Red light (630–660 mμ, $J_{absorbed} = J_{incident} = 5.3$ cmm quanta/min).

| | | |
|---|---|---|
| 10′ Light | −20.0 mm | |
| 10′ Dark | | −20.0 mm |
| 10′ Light | −19.0 mm | |
| 10′ Dark | | −19.5 mm |
| 10′ Light | −20.5 mm | |
| 10′ Dark | | −19.0 mm |
| 30′ Light | −59.5 mm | |
| 30′ Dark | | −58.5 mm |

With aliquot cells, in the same medium, but saturated with 5% $CO_2$ in air, and with the same beam of light, the yield was determined by the 2-vessel method and a quantum requirement of 3 was obtained. This means that in 30′ light the pressure changes that might have been expected above with the NaOH in the side-arm:

| | |
|---|---|
| 30′ Dark | −58.5 mm |
| 30′ Light | −11.5 mm against 59.5 mm obtained. |

*Conclusion:* This experiment shows that light did *not* act on respiration or any intermediates of respiration. When light decreased the negative pressure changes due to respiration, it did so by photosynthesis that compensated the respiration manometrically but not chemically.

SLIDE 17. QUANTUM REQUIREMENT OF $CO_2$ ASSIMILATION AT CONSTANT $O_2$ PRESSURE MAINTAINED BY OXY-BIS(COBALTODIHISTAMINE) (21)

| Pre-illumination (fluorescent white light) Red light (mm³/min) | 0-1 hr 9.2 | | 3-5 hr 4.7 | |
|---|---|---|---|---|
| | Time *min* | h *mm.* | Time *min* | h *mm.* |
| *A.* Fluorescent white light | 15 | − 2 | 10 | − 6 |
| *B.* Fluorescent white + red light | 30 | −34 | 20 | −23.6 |
| *C.* Fluorescent white light | 15 | − 1 | 10 | − 6 |
| mm red light action (B-A-C) | 30 | −31 | 20 | −11.5 |
| mm³ red light action (h × $Kco_2$) | 30 | −71.5 | 20 | −26.5 |
| Quanta red light/$CO_2$ assimilated | $\frac{30 \times 9.2}{71.5} = 3.9$ | | $\frac{20 \times 4.7}{26.5} = 3.6$ | |

300 mm³ Chlorella cells, 7-day culture, in 7 cc medium (pH 5.2). In each vessel side-arm, 0.40 cc 0.43 M $CoCl_2$-0.57 M histamine (pH 7.0). T = 10°C, gas-5% $CO_2/N_2$. $V_{gas}$ = 11.25 cc. $V_{liquid}$ = 7 + 0.8 cc.

$$Kco_2 = ((11.25 \times \frac{273}{283}) + (7.8 \times 1.19) + (0.8 \times 1.19) \times \text{antilog}$$

$$(\text{pH } 7.0\text{–}6.5))/10 = (10.8 + 9.3 + 2.9)/10 = 2.30 \text{ mm}^3$$

with respect to steady state, photosynthetic consumption of $CO_2$ at a constantly maintained pressure of oxygen. Slide 18 provides data for a series of experiments in which the quantum requirement with respect to $O_2$ production and $CO_2$ consumption were determined simultaneously by means of the two-vessel method (3). The results are arranged in order of increasing ratio of $\gamma = CO_2$ consumed/$O_2$ produced, and it is seen that there is a definite tendency for the quantum requirement to decrease as $\gamma$ increases.

SLIDE 18. QUANTUM REQUIREMENT IN RELATION TO ASSIMILATORY QUOTIENT ($\gamma$)

| $\gamma$ | $\gamma = CO_2/O_2$ | $1/\phi$ ($O_2$) | | $1/\phi$ ($CO_2$) | |
|---|---|---|---|---|---|
| | −.80 | 4.6 | | −5.8 | |
| | −.82 | 4.8 | | −5.8 | |
| | −.90[1] | 4.8[1] | | −5.3[1] | |
| −(.8 to 1.0) | −.90[1] | 4.5[1] | 4.5 | −5.0[1] | −5.0 |
| | −.97 | 3.6 | | −3.7 | |
| | −1.00[1] | 4.4[1] | | −4.4[1] | |
| | −1.01[3] | 3.6[3] | | −3.6[3] | |
| | −1.02[2] | 3.5[2] | | −3.4[2] | |
| | −1.03[1] | 2.9[1] | | −2.8[1] | |
| | −1.04[1] | 3.9[1] | | −3.8[1] | |
| −(1.0 to 1.2) | −1.04 | 4.2 | 3.6 | −4.0 | −3.3 |
| | −1.08 | 3.2 | | −3.0 | |
| | −1.10[2] | 3.0[2] | | −2.7[2] | |
| | −1.13[1] | 4.2[1] | | −3.7[1] | |
| | −1.18 | 3.6 | | −3.0 | |
| | −1.23 | 2.8 | | −2.3 | |
| | −1.24[2] | 3.0[2] | | −2.4[2] | |
| −(1.2 to 1.4) | −1.25 | 2.5 | 3.0 | −2.0 | −2.4 |
| | −1.23 | 3.4 | | −2.7 | |
| | −1.33 | 3.2 | | −2.4 | |
| Average | −1.08 | 3.7 | | −3.4 | |

[1] Respiration compensated or overcompensated with unmeasured white light. [2]Respiration overcompensated 6-fold with unmeasured red light. [3]Respiration overcompensated 20-fold with unmeasured white light (dark respiration 65 cmm $O_2$ consumption, total photosynthesis 1300 cmm $O_2$ production/hr/340 cmm cells).

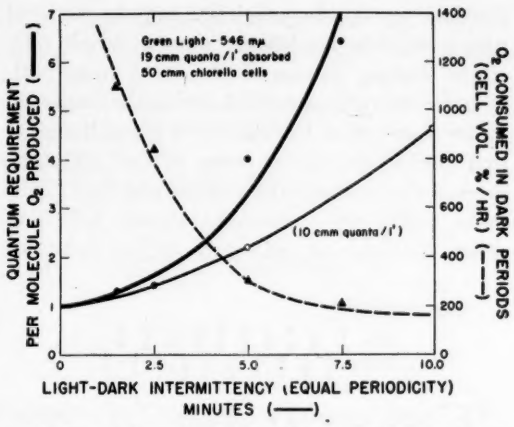
ONE-QUANTUM MECHANISM OF PHOTOSYNTHESIS

From the data presented it can be seen that long-term, steady state photosynthesis may be carried out, under *appropriate* conditions, with nearly perfect quantum efficiency, that is, with a minimum requirement of 3 quanta absorbed/molecule of $O_2$ produced from $CO_2$ and $H_2O$, which approaches closely the theoretical thermodynamic requirement. From this result, notable as it is, it might appear that photosynthesis is no 1-quantum reaction proceeding in accord with Einstein's law of photochemical equivalence.

On the other hand, in the photochemistry of the non-living world, indeed of the non-photosynthetic world, one knows of no 'several-quantum' endothermic reaction taking place, nor can one readily picture the mechanism of such a reaction.

The solution to the unique quantum problem found in photosynthesis has been attained by the discovery of conditions under which it is possible to observe an approximate 1:1 correspondence between $O_2$ produced and quantum absorbed (7 *et seq.*). As indicated in slide 19, this is attained by employing suitably moderate light intensities and an appropriate light-dark intermittency schedule of the order of equal 1-minute light and dark intervals. As the equal-period intermittency is decreased from the hitherto customary periodicity of 5–10 minutes and more, down to about 1 minute (± 30 sec.) the observed quantum requirement decreases to a value of about 1, the quantitative aspects depending upon light intensity. The progressive decrease of quantum requirement, with increasing frequency of intermittency, down to a quantum value of unity corresponds to a greatly increased rate of oxygen production per unit of time at any given light intensity, and is paralleled by a corresponding increase of oxygen consumption, during the dark periods, that may be several times greater than the rate of respiration proper, measured during relatively prolonged intervals of darkness before and after the intermittency schedule. Slide 20 provides an additional illustrative protocol of an experiment with a 1-minute light-dark intermittency and slide 21 represents an idealized, model experiment, with readings indicated for every minute of intermittency.

It is clear that the increased frequency of intermittency schedule has permitted us to make a temporal separation of the true, 1-quantum photochemical reaction in photosynthesis from a rather slow dark reaction whose half-time is the rough order of one minute or somewhat less. In the photochemical reaction $O_2$ is produced and $CO_2$ absorbed, whereas in the dark reaction the converse takes place to nearly but not quite equal extent, with the result that the light reaction (at sufficiently low light intensity!) starts out with a quantum requirement of 1 but after several minutes the back reaction has built itself up sufficiently to greatly lower, and so to speak hide, the true photochemical yield, as illustrated by the data and formulations in slide 22. Upon cessation of illumination the further progress of




Slide 19. LIMITING 1-quantum requirement per $O_2$ produced, and enhanced $O_2$ consumption, in respective light and dark periods of intermittency schedule (8). 50 cmm Chlorella cells/7 cc of medium in each of 2 vessels (volumes, 31.23 and 20.93 cc), pH 4.5, T = 20°C, gas phase 11% $CO_2$ in air, % transmission of measured green light-52% (determined with type of vessel in slide 7). Manometers read without stopping or shaking.

SLIDE 20. QUANTUM REQUIREMENT OF PHOTOREACTION: ILLUSTRATIVE DATA WITH GREEN LIGHT AT 1′ INTERMITTENCY (8)

50 cmm Chlorella cells/7 cc medium per vessel. pH 4.5, temperature—20°C. Gas phase, 11% $CO_2$ in air. Green light, wavelength = 546 mμ. Incident intensity into vessels, 19.7 cmm quanta/min, of which 48% absorbed (transmission, 52%). General overhead illumination (unmeasured intensity), 200 watt incandescent lamp at 30 cm distance.

| | Pressure Changes (mm) | | | |
|---|---|---|---|---|
| | Large Vessel 20.93 cc $K_{O_2}$ = 5.64 mm² | | Small Vessel 16.59 cc $K'_{O_2}$ = 2.82 mm² | |
| Green: | +1′ | −1′ | +1′ | −1′ |
| | 1.0 | 0 | 2.0 | −1.0 |
| | 0.5 | −0.5 | 2.0 | −0.5 |
| | 1.5 | 0 | 2.0 | 0 |
| | 0.5 | −0.5 | 1.5 | −1.0 |
| | 1.5 | −0.5 | 2.5 | −0.5 |
| | 1.0 | −0.5 | 2.0 | −1.0 |
| | 1.0 | −0.5 | 2.0 | −0.5 |
| | 1.0 | 0 | 2.0 | −0.5 |
| | 1.0 | 0 | 2.0 | −0.5 |
| | 1.0 | −0.5 | 2.0 | −0.5 |
| Per 10′: | 10.0 | −3.0 | 20.0 | −6.0 |
| Light Action: | 13 mm (H) | | 26 mm (H′) | |

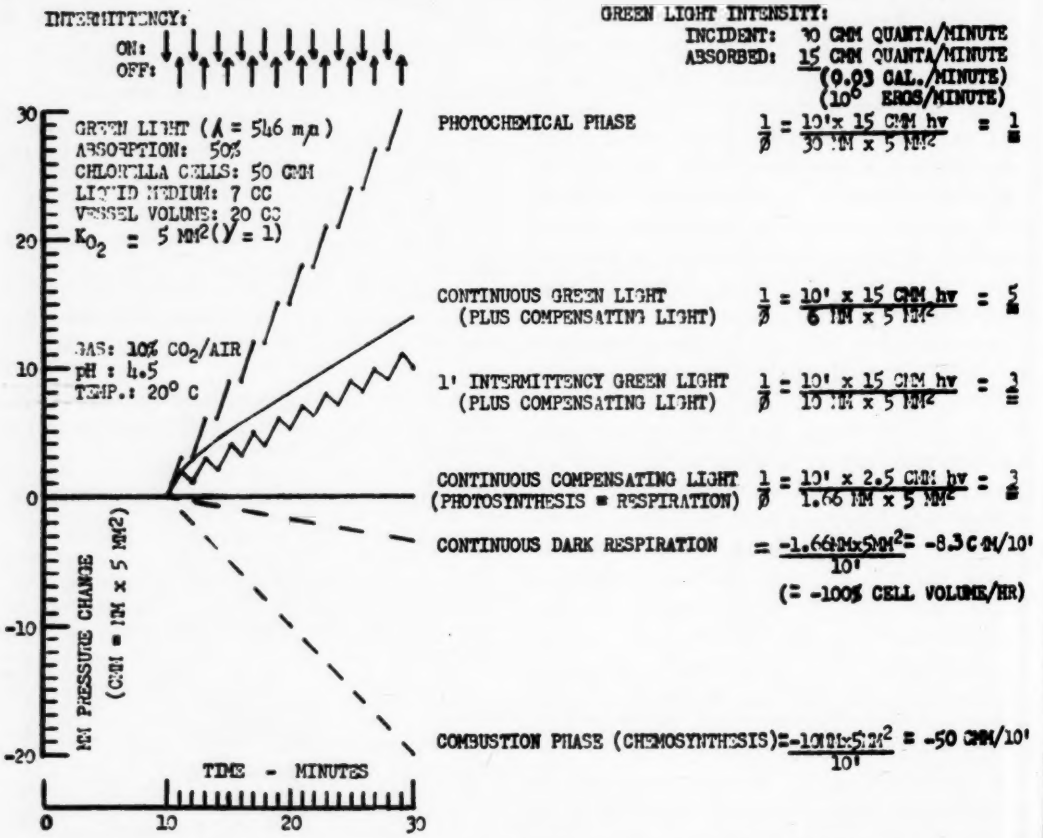
$$H'/H = 26/13 = 2.0$$

$$\gamma = -CO_2/O_2 = -1.11$$

$$\text{Quanta per } +O_2 = \frac{10' \times 19.7 \text{ cmm} \times 0.48}{26 \text{ mm} \times 2.82 \text{ mm}^2} = \frac{95}{73} = 1.3$$

$$\text{Quanta per } -CO_2 = 1.3/1.11 = 1.15$$

the dark reaction to completion may be observed under suitable conditions, among which adequate shaking is one of the most important. It is interesting to note that during the transition phase observed at the start of a given illumination period, the rate is faster at first and then decreases to a steady state value, and that this is quite unlike the commonly known induction phase obtained at relatively higher light intensities, where the rate is less at first, and builds up to a higher, not lower, steady state rate.




Slide 21. MODEL, prototype experiment on effect of intermittent versus continuous light on energy yield and mechanism in photosynthesis.

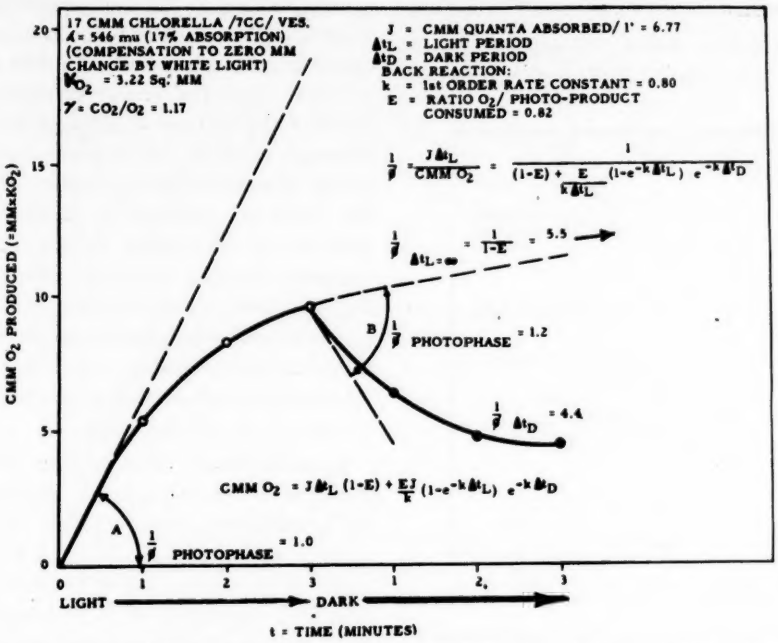
tensities, where the rate is less at first, and builds up to a higher, not lower, steady state rate.

### ENERGY CYCLE OF PHOTOSYNTHESIS

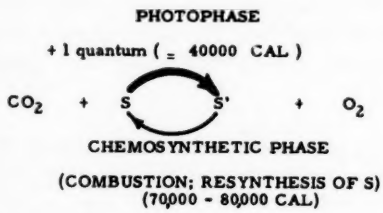
The temporal separation of photosynthesis into a light reaction and a dark combustion reaction, in the manner just outlined, may be represented by the photosynthetic energy transformation cycle pictorialized in slide 23, in which "S" may be taken to include the complex of substances involving chlorophyll in such form as it occurs in the plant leaf and reduced material that *1*) with $CO_2$, requires but 1 quantum of light to produce 1 molecule of $O_2$ and organic product, and *2*) can be reconstituted by means of energy (~70,000 calories) derived from a chemosynthetic process wherein ⅔ or more of a molecule of $O_2$ is burned to yield ⅔ or more of a molecule of $CO_2$. The energy obtained in this chemosynthetic combustion is not given off as heat, but is utilized with high efficiency to reconvert S′ back to S, which is presumably a reductive process.

The net output of photosynthesis is thus a balance between the light reaction and the combustion reaction, so that, for example, if 1 mole quantum of light has produced 1 molecule of $O_2$, and ⅔ to ¾ have reacted back, a net of ⅓–¼ of a molecule of $O_2$ remains; or, correspondingly, 1 (¼) mole of $O_2$ remains when 3 or 4 mole quanta have been absorbed, corresponding to an overall quantum efficiency of 2.7/3 to 2.7/4 or 90–67% respectively, as frequently observed

experimentally under suitable conditions that are easy to obtain if one wishes.


17 CMM CHLORELLA /7CC/ VES.
λ = 546 mu (17% ABSORPTION)
(COMPENSATION TO ZERO MM CHANGE BY WHITE LIGHT)
$K_{O_2}$ = 3.22 Sq. MM
γ = $CO_2/O_2$ = 1.17
J = CMM QUANTA ABSORBED/ 1' = 6.77
$\Delta t_L$ = LIGHT PERIOD
$\Delta t_D$ = DARK PERIOD
BACK REACTION:
k = 1st ORDER RATE CONSTANT = 0.80
E = RATIO $O_2$/ PHOTO-PRODUCT CONSUMED = 0.62
$\frac{1}{\phi} = \frac{J\Delta t_L}{CMM\ O_2} = \frac{1}{(1-E) + \frac{E}{k\Delta t_L}(1-e^{-k\Delta t_L})\ e^{-k\Delta t_D}}$
$\frac{1}{\phi}_{\Delta t_L=\infty} = \frac{1}{1-E} = 5.5$
B $\frac{1}{\phi}$ PHOTOPHASE = 1.2
$\frac{1}{\phi}\ \Delta t_D$ = 4.4
$CMM\ O_2 = J\Delta t_L(1-E) + \frac{EJ}{k}(1-e^{-k\Delta t_L})\ e^{-k\Delta t_D}$
A $\frac{1}{\phi}$ PHOTOPHASE = 1.0
CMM $O_2$ PRODUCED (=MM×$KO_2$)
LIGHT
DARK
t = TIME (MINUTES)

Slide 22. TIME course and quantum requirement ($1/\phi$) of photosynthetic cycle (12, 14, 18).




Slide 23. PHOTOSYNTHETIC energy transformation cycle (14).

The mode of calculation of cycle output is illustrated for an actual experiment in slide 24, making use of the photochemical yield data presented in slide 20, but containing the additional, necessary information concerning the manometric changes occurring before and after the intermittency schedule reported in slide 20. In calculating cycle output, one adds algebraically the pressure changes occurring during any given total period of intermittency schedule of added measured light, and subtracts algebraically from this the average pressure change occurring before and after the schedule for the same given period of total time. In calculating photochemical yield, however, one merely takes the algebraic difference between the pressure changes occurring in the intermittency periods with and without added measured light, without regard to pressure changes occurring before and after the given intermittency schedule. Slide 25 also illustrates, for a range of different light intensities, the difference between the cycle output and the true photochemical yield, which approach approximate values of respectively 3 and 1 at the lowest

SLIDE 24. QUANTUM REQUIREMENT OF PHOTOSYNTHETIC CYCLE OUTPUT (8)

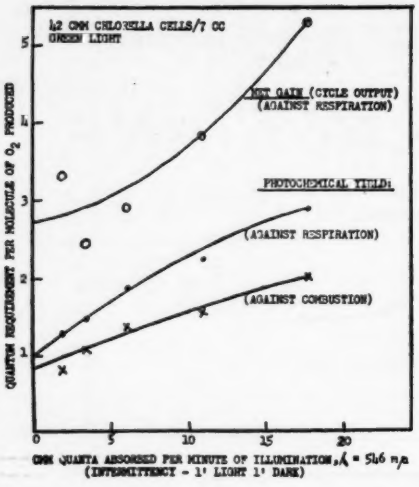
| Intermittency: 1′/1′ | Large Vessel | Small Vessel |
|---|---|---|
| Σ10′ white | − 3.0 mm | − 6.0 mm |
| Σ10′ white + green | +10.0 mm | +20.0 mm |
| Positive pressure in intermittency, 20′ | +7.0 mm | +14.0 mm |
| Continuous white light, positive pressure, per 20′ (before and after intermittency) | +5.0 mm | +7.4 mm |
| Net output, 20′ of intermittency, 1′/1′ | +2.0 mm | +6.6 mm |

$$H'/H = 6.6/2.0 = 3.3. \qquad K'O_2 = 4.5$$
$$\gamma = CO_2/O_2 = -1.3$$

$$\text{Quanta per } +O_2 = \frac{10' \times 19.7 \text{ cmm} \times 0.48}{6.6 \text{ mm} \times 4.5 \text{ mm}^2} = 3.2$$

$$\text{Quanta per } -CO_2 = 3.2/1.3 = 2.5$$

light intensities, but do not attain these low limiting values if the light intensity is too high. The middle curve of slide 25 illustrates that the calculated photochemical yield would have been less, but not greatly less if calculated by using not the $O_2$ consumption (combustion) in the intermittency periods without added measured light, but instead the average $O_2$ consumption (ordinary respiration) observed before and after the intermittency period for the appropriate period of time.


½2 GM CHLORELLA CELLS/7 CC
GREEN LIGHT
NET GAIN (CYCLE OUTPUT)
(AGAINST RESPIRATION)
PHOTOCHEMICAL YIELD:
(AGAINST RESPIRATION)
(AGAINST COMBUSTION)
QUANTUM REQUIREMENT PER MOLECULE OF $O_2$ PRODUCED
GM QUANTA ABSORBED PER MINUTE OF ILLUMINATION, λ = 546 mμ
(INTERMITTENCY - 1' LIGHT 1' DARK)

Slide 25. CYCLE OUTPUT and photochemical yield as a function of light intensity (methodology and conditions approximately the same as in Slides 20 and 24).

Chemosynthesis and photosynthesis thus work together in the assimilation of carbon dioxide in green plant cells. Chemosynthesis furnishes the energy lacking in the light quantum, and then gains back its spent fuel by utilization of the light quantum photochemically. In this way Nature provides that the energy-deficient quanta of visible light can accomplish an overall photochemical reaction that requires three times the energy of a single light quantum. The solution of the quantum problem of photosynthesis thus involves no Maxwellian demon, but instead a chemical auxiliary machine whose specialty is that it drives photosynthesis and is itself driven by photosynthesis. This benign machine perhaps comes as close to being a machine for perpetual motion of the second kind as any that Nature has attempted on a global scale.

**In conclusion:** What greater perfection conformable to the Course of Nature than that, mechanistically, one molecule of chlorophyll absorbs one quantum of light to reduce one molecule of carbon dioxide to produce one molecule of oxygen gas, all in accordance with the Einstein law of photochemical equivalence; and that, thermodynamically, a chemosynthetic energy cycle or machine has been specially devised to attain nearly one hundred per cent efficiency in overall, steady-state, long-term photosynthesis?

It is a pleasure to acknowledge the advice, suggestions, and cooperation of Capt. Jehu Hunter in the preparation of this manuscript.

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# AMERICAN SOCIETY FOR PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

## FALL MEETING

*Madison, Wisconsin, September 8–10, 1952*

## PHYSIOLOGY AND PHARMACOLOGY[1]

WALTER J. MEEK

*From the Department of Physiology, University of Wisconsin School of Medicine, Madison*

IN THE selection of a topic for this brief address, your committee on arrangements must accept much responsibility. They also suffered from the illusion that they had found an after dinner speaker. Although their reasons were doubtless very sound, the lack of encouragement that I received for a strictly scientific talk is something painful to contemplate. Apparently under the topic 'Physiology and Pharmacology' I could make any remarks I might choose, so long as they did not involve cardiac irregularities in various anesthetic states, or the relation of potassium to the same, if any.

You may then without further apology expect some comments on the state of our respective societies, which will be somewhat disconnected and often, I fear, somewhat platitudinous.

One reason for my being here tonight is a very simple one, that of age. All things come to him who waits, even a speech before the pharmacologists.

I became a member of the Federation the year that the pharmacologists held their first formal meeting. Not many of those illustrious founders of your society are with us today. They all seemed to me to be giants in those early days, and my high regard for their ability and vision has never changed.

Individuals who were present at that first meeting are now old, but a society founded as late as 1908 still has all the vigor of its youth. So far as Pharmacology is concerned, I find myself only in the position of a fellow traveler, but the constantly increasing usefulness of this society is a source of great satisfaction to all medical scientists.

With the exception of Anatomy, which is the most ancient of all medical disciplines, Physiology claims motherhood of all the rest. Lest I be accused of prejudice and conceit in this matter, I may quote Claude Bernard who once said, "Physiology is the basis of all medicine."

It was 44 years ago that those interested in the biological effects of chemical compounds organized the American Society of Pharmacology and Experimental Therapeutics. They were all members of the American Physiological Society, a fact I think of really great significance, for the daughter has always greatly resembled the mother and has prided herself on her early home training. These original members were assistant obstetricians at their own birth.

The line between Pharmacology and Physiology is indeed a very thin and tenuous one. It reminds me of the story of Mose who decided that Mandy was destined to be his life partner. Mandy was willing, so Mose went to the county clerk and got the license. However, before it could be put into execution the gorgeous Sarah floated into view and Mose decided he would rather have her. So back he went to the clerk and asked if he could have the license changed. "Sure," said the clerk, "but it will cost you another two dollars." Whereupon Mose said, "Well, that's too bad. As I think it over, I don't believe there is two dollars' difference between those two gals."

I trust, therefore, that it is not unfitting for a mere physiologist to appear on a pharmacological program. Of course, the unabridged dictionary clearly defines what is a physiologist and a pharmacologist, but having lived a good many

[1] Address presented at the dinner of the American Society for Pharmacology and Experimental Therapeutics, September 9, 1952.

years with members of both species, the differences seem to have become less and less.

This similarity of the two sciences is responsible for the frequency with which a physiologist occasionally accepts a chair of Pharmacology or a pharmacologist with even greater ease suddenly becomes a physiologist. Their lineage is after all the same, and although some in each group may raise their eyebrows, this ability to change is a very good thing for both sciences.

The Physiological Society has been a shining example of fecundity. It has never practiced birth control and it has ever believed in a large family. From it, in legitimate fashion, with recorded birth certificates have come the Biochemists, the Pharmacologists, the Experimental Pathologists and the Nutritionists. Almost countless other groups of little brothers and sisters and cousins were originally Physiologists—the Cardiologists; the Bio-physicists; the Neurologists; the General Physiologists; the Endocrinologists; and so on. I mention these for it points out one of the chief functions of Physiology, that of a training ground and stock pile for many varieties of biologists.

Although Physiology is admittedly a science of many facets, many have wondered if it was doomed to disappear as an entity, after so many specialties have been sliced off. But it continues to grow and it has always generously given a seat to the newcomer; like Oliver Hereford, I might say, who once took his young nephew out for a trolley ride. The car was crowded and after getting a seat for himself he was obliged to place the boy on his knee. At the next stop a beautiful blonde got on and grabbed a strap just in front of Mr. Hereford, whereupon he said to his nephew, "Where are your manners, boy, why don't you offer the lady your seat."

My metaphor gets a little mixed, but I do sometimes wonder just what seats Physiology has left to give, as generous as that science seems to be. But one must nevertheless recognize the tremendous increase being constantly made to the sum of knowledge and the segregations that are bound to follow.

Pharmacology, too, is undergoing some of this same kind of fission, at least if the definition of that subject is a study of the action of chemical compounds on living organisms. Toxicology and many divisions of Endocrinology and Immunology are certainly offshoots.

It is obvious that we are in an era of intense specialization, learning more and more about less and less. I fail to see, however, just why a profound knowledge of a specialty should be a term of reproach. It is more likely to be a stage in scientific evolution. At the same time, there is concern about the rigidity of specialization and the fragmentation of our sciences which ensues. Even though the ancient days in which the entire field of knowledge might be made a private domain are gone, with only a nostalgic longing left behind, no one would be so foolish as to deny that some conception of the relationship of things is also a necessity.

Science, as a whole, must accept two lines of effort: one, the duty of discovery which necessitates concentrated application; the other, the understanding what the discoveries mean. We resent the belief in some quarters that the scientist is responsible for the use, good or bad, of a given discovery, but if he is a worthwhile investigator he must at least be able to relate his facts to adjacent fields. The two responsibilities do not always advance on equal fronts. One is slow and painstaking, the other involves no experimental set-up but comes often like a lightning flash after long periods of brooding.

Medicine has followed the modern trend of cleavage into its many parts, but it has, at the same time, been unusually fortunate in preserving a rather marked degree of coherency, of a working relationship among its many subdivisions. Much of this has been due to its overall organizations. I have already referred to the Federation of American Societies for Experimental Biology, of which we are members. In our zeal for using this union for the presentation of our individual experimental reports, we must never overlook its function in holding the parts together and furthering the common understanding and the significance of science as a whole. While in response to the pressure of numbers and the enormous bulk of scientific work, separate meetings such as this seem to be a necessity, I trust they may never crowd out or replace our yearly national meeting in which the various groups catch a glimpse of each other and recognize the common kinship.

It would be interesting in historical fashion to trace the relationship between Physiology and Pharmacology since John Leigh in 1786 published an article entitled "An experimental inquiry into the properties of opium and its effects on living subjects," which is often regarded as the first piece of experimental pharmacology in America. Physiology, dealing with the sequence of events

in the adaptation of an organism to its environment, is a derived science. Its tools are those of chemistry, physics and general biology. Pharmacology, too, is a derived science. It is physiology which has merely chosen a limited field. It is, in brief, the application of physiology to the study of the effect of chemical compounds on living things. Physiology may be considered the trunk of a tree from which specialties branch. It has grown tough with pruning, but the best fruit is always grown on the outer branches.

There are many problems common both to Physiology and Pharmacology. One of the most obvious and important is the selection and insurance of successors. Life in general imposes no such problem. We take what we happen to beget and any attempt at conscious selection seems taboo, but in our world of science, by admission to our courses and by giving or withholding encouragement, we actually select the next generation of physiologists and pharmacologists.

For several years, there has been an urgent demand for professional men and women in our respective fields. In the medical schools for 1950–51 there were staff positions for 393 full time physiologists and for 289 full time pharmacologists. Vacancies at the same time numbered 21 in physiology and 37 in pharmacology. Figures for hospitals, commercial and government laboratories in 1947 indicated a need for 500 pharmacologists in the next 5 years with only 200 probably available. Even at present there are only about 12 departments for training in our universities. Incidentally, it is interesting to know that 65 per cent of all pharmacologists have M.D. degrees, and that the professors are younger than those in any other preclinical group. A survey of the professional status and the future needs of our two sciences, such as the physiologists are now planning, would be very helpful. If we believe that progress in medicine depends on achievement in the basic sciences of medicine, recruitment of first rate material becomes a matter of utmost importance. Not only must physicians be trained but scientists as well, no matter how gifted they may be by nature.

What shall be the criteria for the selection of future workers? No test seems yet to have been devised that will do the job. The history of the use of tests by the Association of American Medical Colleges in choosing medical students has not engendered much enthusiasm. Placing emphasis on speed and a general fund of information might well eliminate many a future genius, for equally or more important are a deep seated love of science, persistence and good judgment, intangibles difficult of measurement. Some attempts to select on the basis of comparative interests have bordered on the ludicrous.

But unless we wish to spend time and money and clog our laboratories with those who are not promising, a choice must somehow be made. After long experience there are many who still think that academic accomplishment remains our best guide, not only in the selection of medical students but of graduate students as well. The average grade cannot be laughed down, for it is a composite estimate made by a good many people. Teachers consciously or unconsciously include in their marks honesty, diligence, love for the work and facility of expression, which makes the estimate all the more valuable. Seldom have I known a promising young research worker who did not come from the upper group of his class. You can't draw out more than you have put in the bank.

Careful selection of graduate material will undoubtedly increase quality but decrease quantity. This, plus the increasing expense of graduate education, might well reduce the output below the normal demand. None of the best must be eliminated, and for this aid must be provided in many cases. We seem to have an abundance of grants, scholarships, and fellowships for all kinds of preparation for specialties, but we have few indeed for the purpose of giving an all round training to young men and women for a life in any one of the basic sciences. Surely our pharmaceutical houses and others who have been so generous in their aid for specific problems would be interested in such an undertaking. Our societies should make active efforts to establish graduate post doctorate fellowships for exceptionally able students who wish to continue in academic life as teachers and research workers.

At the risk of being called a mere pedagogue, which is not very high praise for a scientist, I should like to emphasize a few points in the training of physiologists and pharmacologists. It is not wishful thinking and inner expansiveness that makes a scientist, but a sound knowledge of the basic sciences, chemistry, physics and biology, plus a natural inquisitiveness which cannot be resisted.

Take the beginner at once into your own laboratory and let him learn by example as well

as precept. The enthusiasm that comes to a neophyte when he is first allowed to help in experiments cannot be overestimated.

It is now thought, by many, old fashioned to master a textbook, but I still believe the practice a good one. It gives one a framework on which future knowledge can be hung. As the years go by much will be replaced but a skeleton of truth will always remain to insure an orderly scientific life.

Encourage the reading of scientific papers, classical as well as current. It is sometimes said that too much reading sterilizes the mind, but great men are, I think, invariably great readers. As in the days of Bacon, "Reading maketh a full man."

Encourage a student to keep a notebook in which are recorded all the problems that seem to him worth investigating.

Have him study Claude Bernard's *Introduction to Experimental Medicine*, and learn that there never was a *bad* experiment. Unsuited to our purpose, poorly performed—yes. But always in accord with natural laws.

As soon as possible have him select and outline a problem of his own and turn him loose on it.

Should some such routine be followed, it would soon be clear to any graduate student as well as his professor whether or not the candidate should continue in research.

I have referred to the reading of scientific literature. The coverage of the enormous current output is one of the great individual problems of the scientific workers. Even a modest medical library now adds about 2000 volumes per year to its shelves. Almost any one of these may have something in it of interest to physiologists and pharmacologists. To keep up with the times, one would have to read or look through six volumes per day. So far the only practical solution to such a reading problem has been along the line of abstract and review journals. To cover world literature seems an impossibility, although *Biological Abstracts* is making a worth while and heroic attempt. *Physiological* and *Pharmacolocigal Reviews* are most excellent. *Annual Reviews* also are valuable, and one for Pharmacology might well be considered by the Society.

Scientific work related to the Second World War has had a marked impact on our respective sciences. Although in 1918 medical scientists studied war gases intensively and made suggestions as to the diet of civilians and soldiers, not very much was accomplished and the military authorities were, to put it mildly, generally unreceptive. It was not until the second world conflict that the government realized that the long hairs in their ivory towers held the keys to many problems urgently needing solution. The physicists led the way, but soon physiologists and pharmacologists were serving on committees and commissions of all kinds. The immediate results of this were twofold, and they have continued with us to the present time.

The first was the marvelous release of scientific laboratory workers for travel. Now travel is unquestionably broadening, but there are some doubts about the interminable trips to and fro, unless it can be proved that scientific imagination is stimulated by pullman cars and aeroplanes. A prominent scientist in our own ranks has publicly decried the situation and remarked that the workers had left their shops and were out with the men on horseback in the military camps and elsewhere. I may add that several eminent physiologists were cited in the public press a few days ago as accompanying the big brass on a week's visit to various Army camps, this time of course not on horseback, but in a big Army bomber plane.

In the name of national defense and patriotism it is, I suppose, small wonder that people as sane as are physiologists and pharmacologists should be tempted to fritter away time and energy merely basking in the sunshine emanating from Washington bureaus and commissions. But let us not forget our discoveries are made in the laboratory and not in a committee room. Science must not degenerate into science by remote control.

The second result of directed research during the war years is to my mind much more important. It is the remarkable development of group research. In the application of atomic energy to produce an implement of war, almost innumerable groups were brought into action. The conspicuous success of this undertaking has been responsible in large measure for mass attacks on all kinds of problems. The financing of vast research projects by government and various money collecting organizations, and the consequent opportunity for getting apparatus and research assistants, has now made joint projects in research the rule rather than the exception.

Group research has without question demonstrated its value in the practical application of scientific theory. But, so far as I can find, the

fundamental concepts were not changed in gaining the use of atomic energy. The application, the technology, which I am far from condemning, was the thing obtained by joint mass action. If we, however, give our major attention only to the practical undertakings, granted that they too involve research, where are we to cultivate and secure that rare imaginative leadership which supplies the basis of all science? All applied undertakings stem from the imagination and the discoveries of individual minds.

With apologies to the wit and insight of DuBois, I may say that in the old days a scientist set up his own experiment, recorded his own data, and then went home to a dinner cooked by the maid. After this, he sat down, thought over the next day's work and read something on the subject. Today he has an assistant set up the experiment while he telephones Washington, has another assistant record the data. Then he goes home to a dinner cooked by his wife, after which he washes the dishes. Science by proxy!

What I am really trying to say is that although nostalgia for the good old days will never turn back the clock and we must and will continue to use the methods of group approach, they must never be allowed to destroy the individual thinker on whose documented theories the whole structure depends. The value of chance observations must not be lost because they do not conform to a program. One must not become a slave to a given method. Thinking is just as important as learning.

Medical scientists are now making concerted attacks on cancer, polio, heart disease, and other scourges of mankind, but it should be remembered that even a thousand workers make little headway if the hypothesis they follow is not sound. We speak of the advances we have made, but they are mostly the collection of isolated facts. We need some golden thread of continuity to bind them together and thereby make them understandable and useful. For this we must still look to some man in an ivory tower.

Another weakness of group research that must be guarded against is that it usually offers little general training for the younger worker. The entire problem may be handled by several large, widely separated laboratories. In his own division he may do no more than read a manometer every 10 seconds for a period of hours. This may well dull interest instead of sharpening it.

The cure, I think, is to be sure that each younger worker has time for an individual problem which he alone controls and on which he can expand his powers of observation, enthusiasm, and imagination. This is the road to maturity. In short, modern conditions must not destroy the opportunities for creative work by individuals. Politicians may prate about the principle of individual freedom, but scientists must pay it much more than lip service or degenerate into hacks and find themselves forever unable to enter the promised land of original discovery.

We must never submit to a Commissar of education who tells us to follow a specified scientific line, in a specific way, or else....

Although in this new world, like Alice and the Red Queen, we must run twice as fast to keep up with the country, and I suppose three times as fast to advance, opportunity is still abroad and knocks, not once, but many times at every scientific door. The future really was never more enticing. Stevenson's childhood rhyme remains true; "The world is so full of a number of things—that we should all be as happy as kings."

However, since that was written the world has not been too kind to kings, but it has treated scientists unusually well, even in the face of certain difficulties which speed always entails.

It would be interesting to point out the lines of future development in our two sciences, but prophesying is not a very satisfactory business. Men of genius have not always seen what is to come. Even Harvey little dreamed of the change in all medical thought that was to follow his conception of the circulation. A more modest undertaking would be to mention some of the most promising present day leads. But in this I am preceded by one of our ablest pharmacologists, Dr. Loewi, with whose "Vistas in Pharmacology" I hope you are familiar.

It is easier and safer to make a list of the great discoveries made in the last 50 years as Salter has done. Many items of such a list have not been made by professional pharmacologists, but have been termed "adopted children under the pharmacological roof." I did, however, find a smaller list attributed entirely to pharmacologists. It consisted of:

| | |
|---|---|
| Hormones | Histamine |
| Vitamins and Anti-vitamins | Substitutions such as Demerol |
| Antibiotics | Dicumerol |
| Toxins | Heparin |
| Acetylcholine | |

Now on this list, I have two little complaints. I should like to know just when Baylis, Starling, or Howell became pharmacologists? And we are only a few hundred yards from the building in which Vitamin D was isolated and later made from fatty foods by radiation, and in which the biological results of administrations of Niacin and Dicumerol were first worked out; yet I had not heard before that McCollum, Steenbock, Link and Elvehjem were pharmacologists.

I hear someone remark, "Well they were, only they didn't know it," and this, I think, is an excellent reply. I scarcely need to advise you to watch all corners of the map. Medicine extends from the pig pen to the hospital bed. Pharmacology, young and vigorous, takes information wherever it may be found, works it over for its own therapeutic purposes, and so makes pharmacologists of us all. I have already said you couldn't tell pharmacologists and physiologists apart. Departmentalization is only a matter of convenience and not a prison cell. After all, Science cares little for the label its votaries wear.

Great discoveries in the future as in the past will depend on the application of new types of apparatus and new techniques. My generation recalls with a thrill the advances in cardiac physiology that immediately followed the perfection of the electrocardiograph by Einthoven, in gasteroenterology by Cannon's use of the x-ray and in our knowledge of muscle and nerve by means of the cathode ray tube. Carl Ludwig uttered a great truth when he said that "method is everything," meaning that more and more secrets of nature are unlocked by new devices well used.

No one can tell what the physicists and chemists may invent or discover, but it is safe to say that the x-ray, the internal combustion engine, radio, television, the splitting of the atom and the formation of isotopes are not the end of discovery and human ingenuity. The future of Physiology and Pharmacology, not only in their fundamental researches but in their practical contributions to medicine, depends on our altertness to see and adapt all that is new.

If the definition of Pharmacology is that it is a study of the effect of chemicals on living organisms, it is indeed an ancient science. Shakespeare, who really lived in all ages, hints at this when he quotes the Lord of Ephesus, a supposed contemporary of Pericles, as saying:

"By turning over authorities, I have
Together with my practice made familiar
To me and to my aid the blest infusions
That dwell in vegetatives, in metals, stones."

As a science it has developed along two lines. The first was and is still a qualitative and quantitative study of dose and effect on the entire organism or some special system of such an organism. Although it was Paracelsus who first had any conception of chemical therapeutics, it was not until the time of Blake in 1846 and Crum Brown and Thomas Richard Fraser in 1867 that investigations based on chemical structure got under way. The selection of compounds was pretty much at random and the results, therefore, almost exclusively a matter of chance. The empirical study of the final effects of chemical agents will of course continue, for it has yielded many golden nuggests. However found, even if it is the 606th trial, the discovery of a useful drug will bring fame and emolument to its finder.

Since so many drugs are organic in nature, attempts to relate structure and physiological and pharmacological response had to await the rise of organic chemistry. We still cannot accurately deduce response from structure, but tailor made drugs have been made on occasion, and one may begin to revive our faith that for every disease there is an antidote to be discovered. No sooner was the structure of d-tubocararine determined by King than the significant ammonium compounds were attached to straight chain hydrocarbons. These new methonium compounds proved to be excellent autonomic blocking agents, and offer possibilities in hypertension. The proof that the sulhydryl groups antagonize arsenicals led to the discovery of BAL. The organic chemist and the pharmacologist will share honors in the development of many more active and useful drugs.

Although no one would discount the advances made in Pharmacology by means of Organic Chemistry, Physical Chemistry, Physics and General Biology, the second significant line of research, most active at the present time and pregnant with future meaning, is concerned with the Biochemical approach.

It has been said that the three factors which have rescued Pharmacology from its neglect previous to 1900 are: *1*) the work of Abel, Clark and Hans Meyer; *2*) the rise of Biochemistry; *3*) the development of research in industrial laboratories. Of these, it seems that Biochemistry has been the greatest.

At the moment, Physiology is by many assumed to be limited to the time relation and extent of various pressures, determination of voltages, velocity of the nerve impulse and reflex time and a few examples of simple gas diffusion. To go one micron farther is to fly the flag of Biochemistry.

At present, the Biochemical approach seems to many of us, among the more or less uninitiated, to be largely an enzymic one. In many quarters the words biochemistry and enzymology seem to be almost synonymous. Although we may conceive of a drug replacing some element of the organism by some law of chemical affinity, it is emphasized that in many, possibly the majority of cases, drug action finally involves complicated enzyme systems in the cells. Although beset with many difficulties when it comes to dealing with living cells, cellular physiology and pharmacology will, I think, be one of the most important fields of future research.

I need not labor this audience with a long list of examples, but a few of the utmost significance come to all our minds:

The competition of sulfonamide with para-aminobenzoic acid in the synthesis of folic acid.

The catalytic action of hormones.

Antibiotic inhibition of enzyme systems in parasites.

The growing proof that membrane permeability depends on energy changes in the cell and thus on enzymic processes.

On the lighter side, enzymology explains why the rabbit can eat deadly nightshade with impunity, and best of all how the lowly *bacillus coli*, anticipating I suppose a future antibiotic age, developed himself a penicillinase.

In closing, I should like to pay a tribute to those pioneers who founded our respective societies. Matthew Arnold once said he doubted if science could ever relate its knowledge to the sense of conduct and beauty inherent in man. Had he known the founding fathers of Pharmacology, his doubts might easily have been dispelled.

The part we are now playing in governmental and social activities is a new phase in our development, and offers an opportunity to impress on all that the real pupose of our sciences is a devotion to the common good of mankind. Since the goal of Pharmacology and Therapeutics is to find chemical agents which will benefit the physical being of man, its followers necessarily have peculiar obligations. Not only must they be masters of the experimental method, but they must make every effort to preserve the purity of their preparations, to strive for the proper use of their discoveries, and to prevent false or exaggerated information being peddled to the public. Yet, for the sake of our colleagues, research must not be kept secret. Meeting these situations requires tact and the highest degree of personal integrity.

Amid these difficulties the Pharmacological Society can proceed only by maintaining its high ideals. Its fidelity to these principles and its wisdom in uniting all pharmacologists, once bitterly opposed, has made it one of the most important and influential of the medical sciences. But like political liberty, its freedom in research, its policies and its high aspirations must constantly be kept in mind and fought for. Eternal vigilance is the price of all worthwhile undertakings.

Medicine in our time, with all its inadequacies, has taken on many of the attributes of Deity itself. It has conquered pain, removed plagues and lengthened life. The fates are impotent in its presence. Two of its handmaidens are the Societies of Physiology and Pharmacology.

# AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

## *Symposium on Relationships Between Certain B-Vitamins and Purines and Pyrimidines*[1]

*Chairman:* VINCENT DU VIGNEAUD

# FOLIC ACID AND VITAMIN $B_{12}$ IN THE PHYSIOLOGY OF VERTEBRATES

THOMAS H. JUKES

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RESEARCH in the field of folic acid and vitamin $B_{12}$ in vertebrate physiology probably started with the observations of Minot and Murphy in 1927 that feeding of large amounts of whole liver brought about remissions in pernicious anemia. In retrospect, it appears that these observations were concerned probably with folic acid, since vitamin $B_{12}$ is ineffective orally in pernicious anemia in the doses which would have been supplied by the amounts of liver which were fed. Their later findings, which dealt with the injection of liver extract, were probably related to the effects of vitamin $B_{12}$. These various findings which related folic acid and vitamin $B_{12}$ to the formation of new blood cells foreshadowed the connection between these vitamins and the synthesis of purines and thymine by living organisms, a relationship which was first shown for folic acid in studies with lactic acid bacteria.

The effects of vitamin $B_{12}$ deficiency in animals are not reversed by folic acid. This is somewhat in contrast to the situation in pernicious anemia in human beings, for this disease will characteristically respond to either folic acid or vitamin $B_{12}$ as shown by a return of the blood and bone marrow pictures to normal, although folic acid will not alleviate the nervous involvements which are often associated with this disease. A deficiency of folic acid in vertebrates leads to a macrocytic anemia with attendant characteristic changes in the bone marrow. The anemia is accompanied by leukopenia and the blood dyscrasia has been described in a number of species including human beings, monkeys, pigs, chickens, turkeys and rats. The most completely characterized form of the deficiency in human beings is seen in the megaloblastic anemias of pregnancy and infancy. Typical cases of these anemias do not respond to the injection of ordinary amounts of vitamin $B_{12}$ but a prompt and complete response is uniformly obtained upon the administration of folic acid.

Of great interest to biochemists are the specific functions which have been ascribed to folic acid in metabolism. The interchangeability of folic acid and thymine as growth factors for certain lactic acid bacteria finds its counterpart in the hemopoietic responses in pernicious anemia which may be produced either by small amounts of folic acid or by comparatively massive doses of thymine. Folic acid and vitamin $B_{12}$ are concerned with enabling rats and chicks to respond to homocystine while on a methionine-deficient diet. Studies with folic-acid-deficient rats showed that the deficiency caused a diminution in the incorporation of formate carbon into serine and thymine. A derivative of folic acid, the 'citrovorum factor,' is related to the reversible transfer of the carbon atom from formate to the 2-position in inosinic acid. A deficiency of folic acid slows down the rate of entrance of formate carbon into tissue nucleic acid in rats. The urinary excretion of tyrosine derivatives in scorbutic guinea pigs has been shown to be decreased by the administration of folic acid, and a deficiency of folic acid in chicks and other species specifically reduces the ability of immature females to respond to estrogens as shown by increase in the growth of the oviduct.

Interest in recent years has centered around the chemistry and activity of the 'citrovorum factor,' a biologically functional derivative of

[1] New York City, April 16, 1952.

pteroylglutamic acid. The synthesis of a compound with the properties of citrovorum factor, termed 'leucovorin' by our group and 'folinic acid S.F.' by Shive and co-workers has been described in publications originating from the Lederle and Calco laboratories and from the University of Texas and Lilly Laboratories (1, 2). This compound, 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid, is shown in figure 1. The presence of the CHO group on the 5-position is of especial interest in stabilizing the reduced state of the pteridine ring and in indicating a possible relationship of leucovorin to the transfer of the 'single carbon fragment,' a concept which was first advanced for folic acid by Shive in 1948 and which has been amply reinforced by subsequent experimental observations. Leucovorin crystallizes in the form of white needles. Clinical studies have shown that it produces a typical folic-acid-like response in the megaloblastic anemias. The comparative responses obtained with pteroylglutamic acid and leucovorin in folic-acid-deficient chicks were described by Broquist and co-workers (3). Leucovorin was decidedly less active than pteroylglutamic acid when administered orally and was about half as active as pteroylglutamic acid when given by injection. These phenomena may be related to the action of acid in the stomach of the chick upon leucovorin and to the fact that leucovorin may be a racemic mixture which is only about one-half as active as pteroylglutamic acid for chicks and for *Streptococcus faecalis*.

It has been tempting to speculate that a possible catalytic function of vitamin $B_{12}$ is related to the transformation of folic acid to citrovorum factor, thus providing an explanation for the overlapping effects of folic acid and vitamin $B_{12}$ in pernicious anemia. However, our studies with rats which are illustrated in table 1 indicate that the administration of massive doses of vitamin $B_{12}$ have no effect upon the excretion of citrovorum factor even when pteroylglutamic acid or tetrahydropteroylglutamic acid is simultaneously administered.

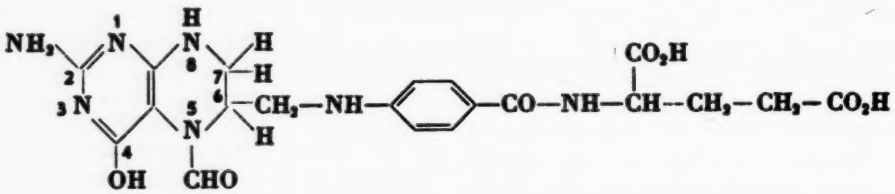


Fig. 1. LEUCOVORIN, 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid.

TABLE 1. FACTORS AFFECTING CITROVORUM FACTOR SYNTHESIS IN RAT AS JUDGED BY URINARY EXCRETION OF CITROVORUM FACTOR

| Group | Supplement injected prior to 20 hr urine collection | Date of urine collection | Total CF in urine |
|---|---|---|---|
| | | | mγ |
| 1334 | None | 7/2/51 | 27 |
| 1334 | 100γ Vitamin $B_{12}$ | 7/6/51 | 24 |
| 1335 | None | 7/2/51 | 13 |
| 1335 | 60γ PGA | 7/3/51 | 432 |
| 1335 | None | 7/5/51 | 30 |
| 1335 | 60γ PGA + 100γ Vitamin $B_{12}$ | 7/6/51 | 355 |
| 1336 | None | 7/2/51 | 26 |
| 1336 | 60γ 4H-PGA | 7/3/51 | 307 |
| 1336 | None | 7/5/51 | 45 |
| 1336 | 60γ 4H-PGA + 100γ Vitamin $B_{12}$ | 7/6/51 | 184 |

Dr. Broquist in our laboratories has found that resting cells of a folic-acid-antagonist resistant strain of *Streptococcus faecalis* which was kindly supplied by Dr. Joseph Burchenal may be used to prepare a system which would enzymatically convert pteroylglutamic acid to citrovorum factor in high yield. The cells were grown on a folic-acid-assay culture medium containing casein hydrolysate and a small amount of Aminopterin, following which the cells were washed, suspended in phosphate buffer and appropriate supplements were added as shown in table 2. After incubation at 37° for 2 hours, the preparation was steamed, centrifuged and citrovorum factor activity was assayed in the supernatant liquid by means of *Leuconostoc citrovorum*. The results are expressed in terms of millimicrograms of natural citrovorum factor, assuming that the natural factor has twice the activity of leucovorin. The production of citrovorum factor activity from pteroylglutamic acid was accelerated by either ascorbate, formate or both. The sum of the effects of these two agents in this experiment was somewhat less

than the activity observed when both were present together; however, in other experiments the sum of the effects taken separately has sometimes been approximately equal to the effect when both were added simultaneously. Serine behaved similarly to formate but glycine and methionine were completely inactive. The effects observed with ascorbate in the presence of either glycine or methionine were no greater than those observed with ascorbate alone. In other experiments, vitamin $B_{12}$ was found to have no effect. The reaction was shown to be enzymatic in nature by the observation that after boiling, the cells were no longer able to produce citrovorum factor activity from formate with or without ascorbate. These experiments strongly imply that pteroylglutamic acid can function as an acceptor of the single-carbon fragment from formate or from serine but it is not yet possible to say whether the citrovorum factor thus formed can in turn donate the formyl group to another metabolite.

Exposure of citrovorum factor preparations to dilute acid at room temperature causes a rapid disappearance of the activity of the preparations tor *Leuconostoc citrovorum*. Certain acid transformation products of leucovorin which are reconverted to leucovorin upon treatment with dilute alkali have been isolated and characterized by Cosulich and co-workers (4). The biological activity of one of these substances, 'anhydroleucovorin-A,' is shown in table 3, in which it was assayed with chicks by comparison with pteroylglutamic acid and leucovorin. The results indicated that anhydroleucovorin was at least as active as leucovorin but both substances were less active than pteroylglutamic acid.

The isolation of a natural form of citrovorum factor from liver has been reported (5, 6) and a comparison of some of the properties of this substance with those of leucovorin have been described by Silverman and Keresztesy (7), who noted that both substances were similar with respect to their ultraviolet absorption spectra and their lability to dilute acid. However, an interesting difference was reported in the biological potencies of the respective acid-treated products for *Streptococcus faecalis*. Both natural citrovorum factor and leucovorin can replace pteroylglutamic acid in the growth of *Streptococcus faecalis*, natural citrovorum factor being about twice as potent as leucovorin in this respect. Upon treatment with acid, the potency of natural citrovorum factor for *Streptococcus faecalis* decreased slightly, while the potency of leucovorin for the same organism increases somewhat. A possible explanation of these phenomena is illustrated in figure 2. Upon reduction of the pteridine ring in the synthesis of leucovorin from pteroylglutamic acid, a new asymmetric center is formed at position 6. This leads to the strong possibility that a racemic mixture of D and L compounds is produced in the synthesis of leucovorin while the second asymmetric center in the glutamic acid radicle of pteroylglutamic acid would be presumably unaffected. Exposure of leucovorin to dilute acid is thought to result in the formation of a bridge between the 5- and 10-positions (4) and Shive and co-workers (8) have suggested that this bridge may open to bring about migration of the CHO group to the 10-position, forming 10-formyl-5,6,7,8-tetrahydropteroylglutamic acid. The reduced pteridine ring is then no longer stabilized by the attachment of a formyl group and is readily oxidized by

TABLE 2. CF FORMATION BY STRAIN OF S. FAECALIS

| Supplements PGA | Ascorbate | Other Additions | Result mγ CF activity |
|---|---|---|---|
| | 5 mg | | 0 |
| | | 5 mg formate | 0 |
| | 5 mg | 5 mg formate | 0 |
| 1γ | | | 20 |
| 1γ | 5 mg | | 125 |
| 1γ | | 5 mg formate | 124 |
| 1γ | 5 mg | 5 mg formate | 350 |
| 1γ | | 10 mg DL serine | 142 |
| 1γ | 5 mg | 10 mg DL serine | 348 |
| 1γ | | 8 mg glycine | <6 |
| 1γ | 5 mg | 8 mg glycine | 95 |
| 1γ | | 15 mg methionine | <6 |
| 1γ | 5 mg | 15 mg methionine | 150 |
| | Boiled cells | | |
| 1γ | | 5 mg formate | 1.2 |
| 1γ | 5 mg | 5 mg formate | 2.8 |

TABLE 3. GROWTH AND HEMOPOIETIC RESPONSES OF PGA-DEFICIENT CHICKS TO PTEROYLGLUTAMIC ACID, LEUCOVORIN AND ANHYDROLEUCOVORIN

| Supplement | Amount injected twice weekly | Results at 28 days Average wt. | Mortality | Hb |
|---|---|---|---|---|
| | | *gm* | % | *gm %* |
| None | | 120 | 80 | 6.8 |
| PGA | 5γ | 214 | 0 | 9.7 |
| PGA | 10γ | 270 | 0 | 10.7 |
| Leucovorin | 7.5γ | 162 | 30 | 8.8 |
| Leucovorin | 15γ | 271 | 0 | 8.8 |
| Anhydroleucovorin | 7.5γ | 213 | 10 | 9.7 |
| Anhydroleucovorin | 15γ | 279 | 0 | 11.4 |

air, thus producing 10-formylpteroylglutamic acid which does not possess an asymmetric center at position 6. As shown in table 4, the natural form of citrovorum factor would undergo a loss of potency in this procedure but leucovorin, containing equal parts of natural biologically-active

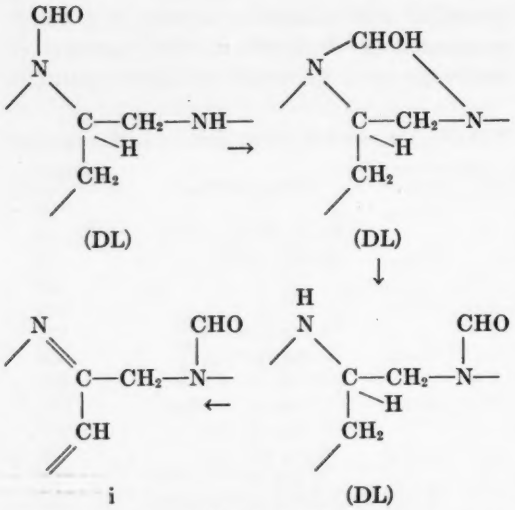


Fig. 2. Possible effects of acid and oxygen upon leucovorin.

Table 4. Possible effects of HCl and $O_2$ on DL-CF (leucovorin)

| Substance | Potency | Substance | Potency |
|---|---|---|---|
| 1 μg D-CF | 0.77[1] → | 1 μg 10-CHO PGA | 0.52 |
| 1 μg L-CF | 0 → | 1 μg 10-CHO PGA | 0.52 |
| *Totals* | | | |
| 2 μg DL-CF | 0.77 → | 2 μg 10-CHO PGA | 1.04 |

[1] Activity measured for *S. faecalis* in terms of PGA.

Table 5. Results with 'X-methyl PGA'

| Level of antagonist | Level of PGA | Ratio |
|---|---|---|
| 0.3[1] | 0.01[2] | 30 |
| 2.0[1] | 0.1[2] | 20 |
| 30[1] | 1.0[2] | 30 |
| 1,000[3] | 3[4] | 333 |
| 10,000[3] | 10[4] | 1000 |

[1] μg/10 ml in medium for *S. faecalis*. [2] Amount of PGA 'blocked' by antagonist. [3] Mg/kg of purified diet for rats. [4] Amount of PGA needed to reverse antagonist.

citrovorum factor and of the unnatural biologically-inactive enantiomorph would give rise to two molecules of the biologically-active substance 10-formylpteroylglutamic acid, thus resulting in an increase in potency for *Streptococcus faecalis*.

Recent studies with the citrovorum factor have made it possible to interpret a number of findings which have been made regarding the biological actions of synthetic analogues of pteroylglutamic acid, the "folic acid antagonists." It is now possible to divide these substances into two classes. The first group, which are exemplified by 9-methylpteroylglutamic acid, have the following general characteristics: *a*) They are competitively reversed by pteroylglutamic acid in experiments with animals; *b*) the inhibition index for *Streptococcus faecalis* is proportional to the concentration of the antagonist so that the index remains roughly constant with increasing concentrations of the antagonist; *c*) these analogues produce no effect on *Leuconostoc citrovorum* in the presence of citrovorum factor; *d*) the substances in this class block the formation of citrovorum factor from pteroylglutamic acid but they do not interfere with the utilization of citrovorum factor. Some of these points are illustrated in table 5.

The second class of antagonists is typified by Aminopterin and includes various 4-amino derivatives of pteroylglutamic acid. These substances are used clinically to give temporary relief of acute leukemia in children, and their pharmacology has been extensively described by Philips and Thiersch (9). In experiments with animals, the reversibility of these antagonists by pteroylglutamic acid is negligible or slight. Their inhibition index for *Streptococcus faecalis* increases markedly with the concentration of the antagonist and appears to be approximately proportional to the logarithm of the concentration of the antagonist. The substances in this class show competitive reversal by citrovorum factor when studied with *Leuconostoc citrovorum*. Finally, these antagonists, as originally shown by Nichol and Welch (10), block the formation of citrovorum factor from pteroylglutamic acid. In addition, these antagonists block the utilization of citrovorum factor competitively as shown by our experiments with mice and with *Leuconostoc citrovorum*. Some of these points are illustrated in table 6.

Table 7 shows a comparison between antagonists of the two classes in studies with *Streptococcus faecalis* and simultaneously with *Leuconostoc citrovorum*. Both 4-aminopteroylglutamic acid (Aminopterin) and 10-methylpteroylglutamic acid are strongly antagonistic towards pteroylglutamic acid when studied with *Streptococcus faecalis*. Aminopterin also reverses the

growth-promoting effect of both pteroylglutamic acid and citrovorum factor for *Leuconostoc citrovorum*. In contrast, 10-methylpteroylglutamic acid is only weakly antagonistic against pteroylglutamic acid for *Leuconostoc citrovorum*, no doubt corresponding to the very limited amount of conversion of pteroylglutamic acid to citrovorum factor which this organism is able to undertake and 10-methylpteroylglutamic acid is without effect on citrovorum factor when studied with *Leuconostoc citrovorum*. These findings are represented diagrammatically in figure 3. We have found that tetrahydropteroylglutamic acid

TABLE 6. RESULTS WITH 4-AMINO-10-METHYL PGA

| Level of Antagonist | Level of PGA | Ratio |
|---|---|---|
| 0.06[1] | 0.01[2] | 6 |
| 0.2[1] | 0.1[2] | 2 |
| 1.0[1] | 1.0[2] | 1 |
| 8.0[1] | 10.0[2] | 0.8 |
| 1[3] | 0.1[4] | 10 |
| 3[3] | 100[4] | 0.03 |
| 10[3] | 0[5] | very low |

[1] μg/10 ml in medium for *S. faecalis*. [2] μg PGA 'blocked' by antagonist. [3] Mg/kg of purified diet for rats. [4] Mg PGA needed to reverse antagonist. [5] No reversibility at highest level of PGA fed.

TABLE 7. REVERSAL OF TOXICITY OF FOLIC ACID ANTAGONISTS BY PTEROYLGLUTAMIC ACID (PGA) AND LEUCOVORIN (CF)

| Antagonist | Growth (optical density) *S. faecalis* 0.1γ PGA | *Le. citrovorum* 0.1γ PGA | *Le. citrovorum* .002γ CF |
|---|---|---|---|
| 0 | 1.22 | 1.60 | 1.65 |
| 0.03γ 4-$NH_2$PGA | 0.37 | 1.43 | 1.65 |
| 0.1γ 4-$NH_2$PGA | 0.20 | 0.47 | 1.65 |
| 0.3γ 4-$NH_2$PGA | 0 | 0 | 1.41 |
| 1.0γ 4-$NH_2$PGA | 0 | 0 | 0.30 |
| 0.1γ 10-$CH_3$PGA | 0.02 | 1.60 | 1.65 |
| 1.0γ 10-$CH_3$PGA | 0 | 1.60 | 1.65 |
| 10γ 10-$CH_3$PGA | 0 | 1.40 | 1.65 |
| 100γ 10-$CH_3$PGA | 0 | 0.39 | 1.68 |

behaves similarly to leucovorin in the presence of Aminopterin when studied with mice (table 8) and with *Leuconostoc citrovorum*. Leucovorin and, at somewhat higher levels, tetrahydropteroylglutamic acid and anhydroleucovorin, were all effective in reversing the toxic effects of Aminopterin for mice. In contrast, 10-formylpteroylglutamic acid was ineffective in reversing the antagonist, in spite of the fact that 10-formylpteroylglutamic acid, as originally noted by Shive (11), is somewhat more effective than pteroylglutamic acid in reversing the effects of certain antagonists for lactic acid bacteria.

It thus becomes possible to visualize the conversion of pteroylglutamic acid to citrovorum factor as taking place in two steps, the first of which, the reduction of pteroylglutamic acid to

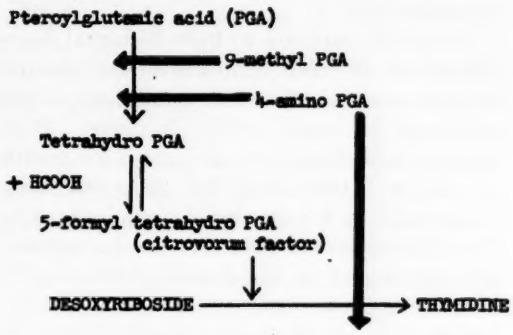


Fig. 3. EFFECT of antagonists on transformation of PGA to CF and on its utilization.

TABLE 8. REVERSAL OF TOXICITY OF AMINOPTERIN (A.) IN MICE BY LEUCOVORIN AND RELATED COMPOUNDS

| Group no.[1] | Supplement (Injected 3x weekly) | Wt. change and survivors ( ) at 6 days | 8 days |
|---|---|---|---|
| 1 | 0 | 2.5(12) | 3.8(12) |
| 2 | 10γ A. | —4.7(6) | all dead |
| 3 | 10γ +30γ 10-CHO-PGA | —2.2(3) | all dead |
| 4 | 10γ +100γ 10-CHO-PGA | —3.9(3) | all dead |
| 5 | 10γ +30 4H-PGA[2] | 1.5(10) | 1.3(12) |
| 6 | 10γ +100γ 4H-PGA[2] | 3.1(10) | 3.3(12) |
| 7 | 10γ +30γ anhydro-L[3] | 2.3(12) | 3.5(12) |
| 8 | 10γ +100γ anhydro-L[3] | 3.4(12) | 3.6(12) |
| 9 | 10γ +10γ leucovorin | 0.1(12) | 0.3(12) |
| 10 | 10γ +20γ leucovorin | 2.2(12) | 3.5(12) |

[1] Twelve mice per group. [2] 5,6,7,8-tetrahydro PGA. [3] Anhydroleucovorin.

TABLE 9. RESPONSE OF $B_{12}$-DEPLETED RATS TO HOMOCYSTINE, BETAINE AND METHIONINE AS RELATED TO OTHER DIETARY SUPPLEMENTS

| Addition per kilo of basal extracted-pea diet 3-A | Growth results at 26 days[1] (gm.) Without $B_{12}$ Wt. | Response | With $B_{12}$ Wt. | Response | Increase in response due to $B_{12}$ |
|---|---|---|---|---|---|
| None | 51 | | 93 | | |
| 6 gm homocystine | 45 | —6 | 118 | 25 | 31 |
| 2 gm betaine | 51 | 0 | 92 | —1 | —1 |
| Homocystine + betaine | 76 | 25 | 144 | 51 | 26 |
| 3 gm methionine | 86 | 35 | 120 | 27 | —8 |
| Methionine + betaine | 82 | 31 | 123 | 30 | —1 |

[1] 7 rats per group.

the 5,6,7,8-tetrahydro form, may well be brought about by ascorbic acid. The second step, the introduction of the formyl group may possibly be carried out by formic acid or serine.

In lactic acid organisms, one function of citrovorum factor is related to the formation of thymidine. In this reaction, citrovorum factor is competitively inhibited by Aminopterin and this inhibition is reversed non-competitively by thymidine (12).

Vitamin $B_{12}$ appears to have biological functions which are closely related to those of pteroylglutamic acid and the citrovorum factor in the utilization of single carbon fragments. Both vitamin $B_{12}$ and folic acid are needed for growth of rats on 'labile-methyl'-free diets containing homocystine as a precursor of methionine (13). The addition of methionine to such diets enables the rats to grow in the absence of vitamin $B_{12}$ and folic acid, and choline or betaine will enable rats to grow on these diets in the absence of methionine and without providing supplementary vitamin $B_{12}$. Evidently, the utilization of certain sources of the methyl group of methionine, perhaps serine and glycine, for the methylation of homocystine, is greatly improved by supplementary vitamin $B_{12}$ and folic acid. Similar findings have been made with vitamin-$B_{12}$-deficient chicks in our laboratory. It was found that these animals show a growth response to methionine but not to homocystine, with or without supplementary betaine or choline, when placed on a basal diet deficient in methionine and containing supplementary homocystine. In contrast, when the chicks received vitamin $B_{12}$, a growth response was obtained to either methionine of homocystine. Some of our recent findings with rats (14) are summarized in table 9. The animals were obtained from mothers who had been kept on a diet deficient in vitamin $B_{12}$ during the last ten days of pregnancy and during the lactation period. A basal diet containing 70% of alcohol-extracted dried peas was used, on which the rats showed a growth response to homocystine plus betaine, to methionine, or to methionine plus betaine but not to homocystine or betaine alone. However, when the diet was supplemented with 50 $\mu$g vitamin $B_{12}$/kg, a growth response was obtained to homocystine.

Various investigations in vertebrate nutrition have related vitamin $B_{12}$ to the metabolism of high levels of dietary protein, to the formation of purine and pyrimidine desoxyribosides, to the growth response to choline and betaine in chicks, to the prevention of fatty livers in rats and to the maintenance of the central nervous system in human beings with pernicious anemia (15).

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# B-VITAMINS INVOLVED IN SINGLE CARBON UNIT METABOLISM

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ONLY recently several different approaches to the metabolism of single carbon units have become closely interrelated. Investigations concerning the nutritional requirements of animals for 'labile methyl' groups, transmethylation, the utilization of isotopically labeled carbon atoms in various compounds as sources of single carbon units, and the biochemical functions of B-vitamins involved in single carbon unit transfer represent independent approaches to the general problem of the metabolism of single carbon units. Competitive analogue-inhibitors of B-vitamins have played a major role in investigations concerning the function of B-vitamins in reactions involving single carbon units.

## FUNCTION B-VITAMINS INVOLVED IN SINGLE CARBON UNIT METABOLISM

*p-Aminobenzoic Acid.* Subsequent to Woods' discovery that *p*-aminobenzoic acid competitively prevents the toxicity of sulfonamides for certain organisms (1), methionine was found to reverse the toxicity of sulfonamides over a limited range of concentrations (2–5). The effect was enhanced by purines which also exert antisulfonamide effects for microorganisms *in vitro* and *in vivo* (6, 7). While numerous theories for the mode of action of such substances were suggested including the possibility that these substances are end products of reactions catalyzed by *p*-aminobenzoic acid, the reversing actions of such compounds were at times cited as evidence against the competitive analogue-metabolite theory of sulfonamide inhibition.

However, the development of theoretical aspects of competitive analogue-metabolite inhibitions has resulted in testing techniques which offer proof for the mode of action of reversing agents of a competitive inhibitory analogue (8–11). Application of such an analysis to sulfonamide inhibition has resulted in elucidation of the interrelationships depicted in figure 1 (12, 13). Sulfanilamide is depicted as inhibiting a reaction involving the conversion of *p*-aminobenzoic acid to an intermediate involved in formation of a coenzyme(s). This coenzyme(s) is required by several apoenzymes each of which catalyzes a specific reaction. Under conditions of sulfanilamide inhibition, the rate of coenzyme synthesis becomes the limiting reaction of the biological system. Of the various apoenzymes competing for the deficient concentration of coenzyme, one particular enzyme system depending upon its apoenzyme concentration and its relative affinity for the coenzyme would become limited by lack of coenzyme sufficiently to retard the response of the entire biological system. In *Escherichia coli*, this first limiting enzyme system is involved in the biosynthesis of methionine. Since the rate of coenzyme formation is determined approximately by a ratio of the concentrations of sulfanilamide to *p*-aminobenzoic acid rather than by the actual concentration of the inhibitor, the rate of coenzyme synthesis necessary for a defined inhibition is constant and corresponds to a constant ratio of inhibitor to vitamin. This ratio has been termed the inhibition index. If methionine is supplied to the medium of *Escherichia coli*, the enzyme system involved in its formation is no longer essential so that inhibition of the system does not inhibit growth of the organism. However, if the ratio of the concentration of the inhibitor to vitamin is increased to such an extent that the greater coenzyme deficiency reduces the activity of another enzyme system to a level insufficient for growth promotion, the organism is again inhibited, but a higher inhibition index corresponding to this new system is obtained. Thus methionine supplements increase the inhibition index by exerting a sparing effect on the coenzyme. In this way, methionine, purines, serine and thymine (or thymidine) exert their effects in the order listed by increasing the inhibition index only if all of the preceding products as listed are present. Since homocysteine does not replace

methionine, it appears that the methylation step is inhibited.

Under these conditions of limiting purine synthesis, 5-amino-4-imidazolecarboxamide accumulates in the medium (14, 15), and the yield of the amine is related to the concentration of glycine available to the organism (16). Although the amine replaces purines for several organisms such as *Lactobacillus arabinosus* (11), it is not utilized by many organisms some of which accumulate the amine in the presence of sulfonamides. Thus, it is likely that a conjugated form of the amine such as the riboside, ribotide, etc., represents the normal intermediate from which the free amine is derived.

Since serine, though interchangeable with glycine for a mutant strain of *Escherichia coli* (17), does not replace glycine as a precursor of the amine, this interconversion appears to be inhibited by sulfonamides (16). Also, serine increases the inhibition index when both methionine and purines are present; consequently, serine appears to be a product of the functioning of *p*-aminobenzoic acid (13).

Thus, *p*-aminobenzoic acid functions in the conversion of homocysteine to methionine, 5-amino-4-imidazolecarboxamide or a derivative to purines, and the interconversion of glycine and serine. All of these functions involve the incorporation of a single carbon unit, and since the 2-carbon of purines was known to be derived from formate (18), the function of *p*-aminobenzoic acid in the incorporation of such a unit was indicated.

In the presence of methionine, purines and serine, but not in the absence of any of these products, thymine or thymidine increases the inhibition index (13). The incorporation of a single carbon unit into thymine was thus anticipated and has been recently confirmed by data indicating the incorporation of radioactive carbon from β-$C^{14}$-serine, of α-$C^{14}$-glycine and of $C^{14}$-formate into the 5-methyl group of thymine (19, 20).

For *Escherichia coli*, a series of end products of the functioning of *p*-aminobenzoic acid exert their effects in a definite order; however, the order in which such products exert their effects

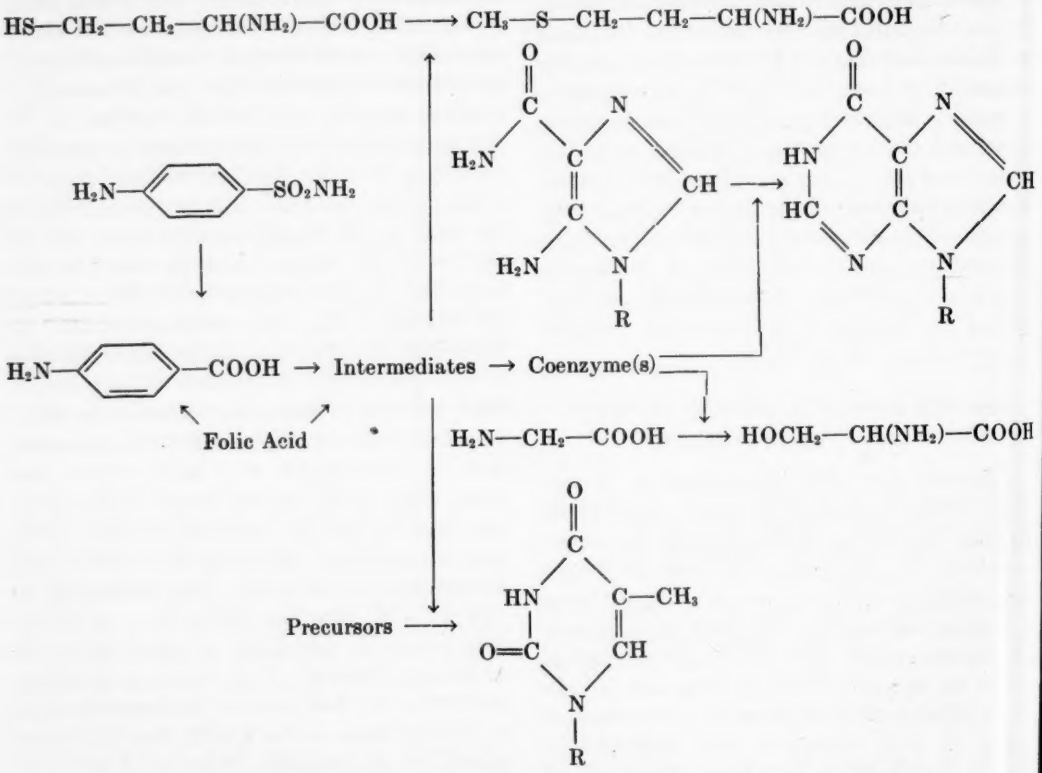


Fig. 1. INTERRELATIONSHIPS and functions of p-aminobenzoic acid and folic acid.

may vary from one organism to another or may be similar to the sequence in *Escherichia coli* (21). Different strains of the same organism may have some variations in the order in which the end products exert their effects.

In growth experiments with organisms requiring *p*-aminobenzoic acid for growth, purines have been reported to have a sparing effect on the requirement (22) and a mixture of amino acids, purines and thymine replaces the *p*-aminobenzoic acid requirement of a mutant strain of *Escherichia coli* (23) and other organisms (24). Thus, similar results are obtained whether the deficiency of *p*-aminobenzoic acid coenzyme(s) is induced by inhibitors or results from genetic deficiencies.

*The Folic Acid Group.* Before the structure of folic acid was known, a combination of both thymine (25) and purines (26) was reported to replace folic acid in the nutrition of certain microorganisms. Since this occurs without production of detectable amounts of folic acid, it was suggested that folic acid functioned in the biosynthesis of purines and thymine (27). For *Lactobacillus casei*, purines and then thymine in an amino acid medium increase the inhibition index obtained with x-methylfolic acid as a competitive inhibitor of folic acid (28), while for *Streptococcus faecalis R* in a similar medium, purines increase the inhibition index, and thymine in the presence of purines completely prevents the toxicity of the inhibitor (29).

Only in the presence of relatively high concentrations of folic acid are serine and glycine interchangeable for promoting growth of *Streptococcus faecalis R*; otherwise in the presence of lower concentrations of folic acid or upon replacement of folic by thymine and purines, both serine and glycine are required by the organism (30). Also, folic acid deficient rats incorporate only about ¼ as much radioactive carbon from $C^{14}$-formate into the β-carbon of serine as normal controls (31).

Recently, the utilization of homocysteine in place of methionine has been shown to be dependent upon adequate supplements of folic acid for rats (32, 33). Thus, folic acid functions in the same reactions as *p*-aminobenzoic acid.

Even before the structure of folic acid was known, sulfonamides were reported to decrease the production of microbiologically active forms of folic acid (34). When the structure of folic acid, which contains a *p*-aminobenzoyl group in combination with a pteridine and glutamic acid, was determined, it became apparent that *p*-aminobenzoic acid was a precursor of folic acid.

Such a concept was supported by evidence that a few organisms utilize folic acid as effectively as *p*-aminobenzoic acid, and for these organisms folic acid prevents non-competitively the toxicity of sulfonamides (35, 36). However, most organisms do not utilize folic acid as effectively as *p*-aminobenzoic acid either in promoting growth of organisms requiring *p*-aminobenzoic acid or in reversing the toxicity of sulfonamides. In fact, most organisms which utilize *p*-aminobenzoic acid in such a manner either do not utilize folic acid or utilize it in a manner suggesting prior conversion to *p*-aminobenzoic acid (35–39).

Since the end products of the functioning of folic acid in organisms requiring folic acid but not utilizing *p*-aminobenzoic acid are identical with those of *p*-aminobenzoic acid in organisms which utilize *p*-aminobenzoic acid but not folic acid, it appears that both *p*-aminobenzoic acid and folic acid function in the formation of the same coenzyme(s) in different organisms. It appears likely that folic acid either is not the normal intermediate in the conversion of *p*-aminobenzoic acid to its coenzyme form or does not penetrate per se to the site of utilization. The first possibility is depicted in figure 1, which indicates two possible routes of utilization of folic acid. One route consists of cleavage of folic acid to *p*-aminobenzoic acid or a related derivative, the utilization of which is prevented competitively by sulfonamides. The other route involves the direct formation of a coenzyme intermediate which is beyond the point at which sulfonamides exert their inhibitory effect. The inability of certain pathogenic organisms to utilize folic acid adequately by the second route provides the basis for their inhibition by sulfonamides in a host which utilizes folic acid for the same enzymatic functions as are inhibited by sulfonamides in the invading organism.

Not only must an organism susceptible *in vivo* to sulfonamides be incapable of utilizing folic acid at the same concentration as the host, but it must not be able to obtain all the end-products of the functions of *p*-aminobenzoic acid, from the host cell, even though the host cell is undoubtedly synthesizing these products with enzyme systems utilizing the same coenzyme(s) which is deficient in the infecting organism but is derived from folic acid in the host. Permeability factors in-

cluding destruction during migration of forms of the products utilized by the pathogenic organism may play a role in allowing such a selective differentiation.

*The Folinic Acid Group.* As part of a program concerning the anti-pernicious anemia factors of liver, the possible existence of a form of folic acid which would be utilized more effectively than folic acid was considered. A group of related factors in liver extracts and other natural sources was found to be more effective than folic acid in reversing the toxicity of x-methylfolic acid for $N^{10}$-formylfolic acid as a possible functional derivative of folic acid (44). Since the $N^{10}$-formyl derivative was considerably more active than folic acid in reversing the toxicity of x-methylfolic acid for *Streptococcus faecalis R*, it appeared likely that folinic acid might be related to the formyl derivative. By reducing $N^{10}$-formylfolic acid, and heating the reduced derivative, an active principle identified as $N^5$-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid-SF, leucovorin) is formed (45–49). This factor is also formed by direct formylation of 5,6,7,8-tetrahydrofolic

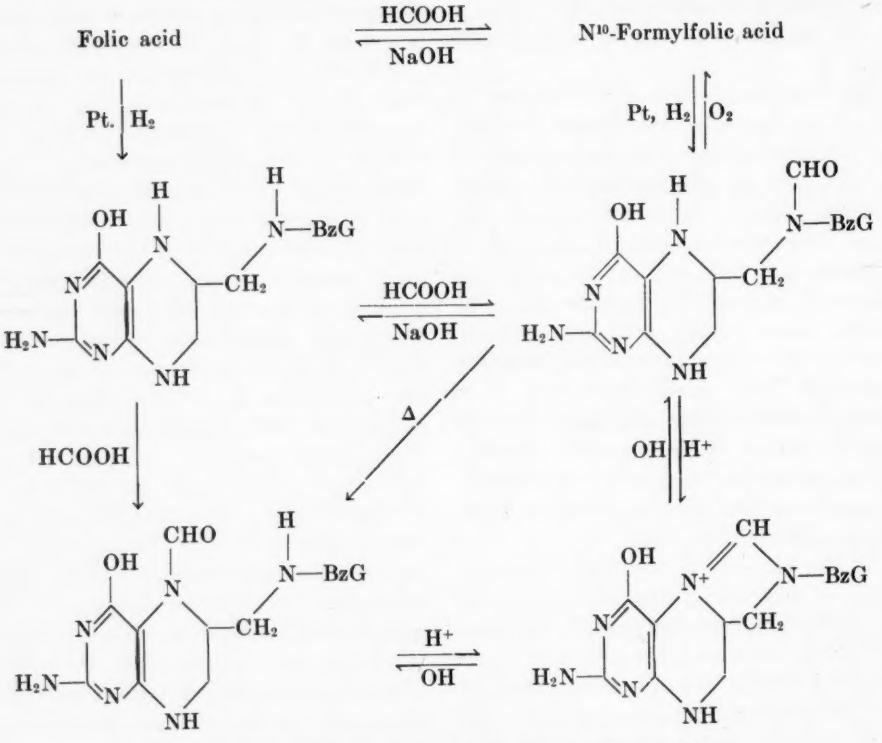


Fig. 2. INTERCONVERSIONS of folic acid and folinic acid-SF.

both *Lactobacillus casei* and *Streptococcus faecalis R* (40). The natural factor closely related to folic acid, termed folinic acid, is 15 times as active as folic acid in preventing the toxicity of the analogue for *Lactobacillus casei* and from 100 to 1000 times as active, depending upon the conditions, for *Streptococcus faecalis R*. The growth of *Leuconostoc citrovorum* is also stimulated by the factor (41, 42), and for this organism thymidine exerts a very marked sparing action upon the requirement for folinic acid (42).

The structure or rhizopterin, $N^{10}$-formylpteroic acid (43), earlier had led to the preparation of acid as indicated in figure 2. Folinic acid-SF is destroyed in acidic solutions with the formation of an imidazolinium compound. The imidazolinium compound is unstable in neutral or alkaline solutions forming $N^{10}$-formyltetrahydrofolic acid which, under anaerobic conditions, rearranges to the $N^5$-formyl derivative or, under aerobic conditions, oxidizes very rapidly. An end product of the oxidation is $N^{10}$-formylfolic acid. These interrelationships are indicated in figure 2.

The natural factor isolated from liver is approximately twice as active as the synthetic

factor, has the same ultraviolet absorption spectra, and undergoes the same type of destruction in acid solution with the formation of an imidazolinium derivative (50, 51). The creation of a new asymmetric carbon during the reduction of the pteridine nucleus would result in the possibility that the synthetic factor may be a mixture of two diasterioisomeric forms, one of which is the active form. Treatment with acidic solutions and assay of the resulting solutions in neutral medium would result in considerable oxidation of the nucleus with the destruction of the new asymmetric center. While this would explain data based on differences in activities before and after acid destruction of the natural and synthetic factors (51, 52), the possibility of small structural differences cannot be excluded.

Although folinic acid replaces the *p*-aminobenzoic acid requirement for at least one organism which does not utilize folic acid (53), folinic acid does not represent a form of folic acid utilized by all such organisms.

*Vitamin $B_{12}$.* One of the assays which was originally developed for the antipernicious anemia principle of liver was based on the ability of the factor to prevent the toxicity of sulfanilamide for *Escherichia coli* in such a manner that the inhibition index obtained with *p*-aminobenzoic acid was increased 3-fold under conditions such that the synthesis of any one of the end-products (methionine, purines, serine or thymine) was limiting the growth of the organism (11). Such data indicates that the factor which was later identified as vitamin $B_{12}$ either is involved in the conversion of *p*-aminobenzoic acid to its coenzyme form or performs a separate function in the incorporation of single carbon units in the methylation step of the biosynthesis of methionine, in the biosynthesis of purines, in the interconversion of glycine and serine, and in the biosynthesis of thymine (11).

The vitamin $B_{12}$ requirement for growth of *Lactobacillus lactis*, and related organisms can be replaced by a combination of thymidine (54, 55) or other desoxyribosides (11, 56–58) with purines or their derivatives (59). Slow growth of *Lactobacillus leichmanni* in the absence of both folic acid and vitamin $B_{12}$ is stimulated by thymidine but not by thymine; however, thymine permits slow growth of the organism in the absence of folic acid but in the presence of vitamin $B_{12}$ (59). Thus, it is apparent that folic acid is more closely associated with the biosynthesis of the thymine moiety and vitamin $B_{12}$ with the desoxyriboside moiety of thymidine. In view of the sparing effect of thymidine upon the folinic acid requirement of *Leuconostoc citrovorum 8081*, the possibility of the involvement of thymidine in the conversion of folinic acid to its coenzyme form is suggested.

## MECHANISM OF REACTIONS INVOLVING SINGLE CARBON UNITS

*Methionine.* The biosynthesis of methionine from homocysteine has been recently found to occur with the incorporation of formate (60–62). The involvement of *p*-aminobenzoic acid and folic acid as well as vitamin $B_{12}$ in this conversion led to studies concerning the naturally occurring reversing agents for sulfanilamide inhibition of *Escherichia coli* under conditions of limiting methionine synthesis. The possibility of a heat-labile derivative of *p*-aminobenzoic acid as a normal intermediate in the biosynthesis of the coenzyme was considered, and a heat-labile reversing agent was discovered in cabbage juice (50, 63). The behavior of the natural factor suggested the possibility that the factor was a sulfonium derivative of methionine. A synthetic factor, 3-amino-3-carboxylpropyldimethylsulfonium chloride (the methyl sulfonium derivative of methionine) has biological activities corresponding to the natural factor and has the same $R_F$ values on paper chromatographs in numerous solvents. On heating, both the natural and synthetic factors decompose to form an amino acid which, on the basis of paper chromatographs in several solvents, appears to be homoserine. The occurrence of the heat-labile factor in natural extracts corresponds closely to that reported for vitamin U, a heat-labile anti-ulcer factor (64).

Since the methyl sulfonium derivative of methionine is approximately twice as active as methionine in reversing sulfanilamide toxicity for *Escherichia coli*, the possibility of a transfer of a methyl group to homocysteine to form two equivalents of methionine exists. However, some mutant strains of *Escherichia coli* requiring methionine do not effectively utilize the compound, and many organisms requiring methionine for growth either do not utilize the compound or utilize it less effectively than methionine.

*Purines.* Many organisms inhibited by sulfonamides or folic acid analogues do not accumulate 5-amino-4-imidazolecarboxamide under conditions of limiting purine synthesis. *Lactobacillus arabinosus*, which is stimulated during short incubation periods in its growth by the amine or by

purines, is such an organism. Not only does the amine not accumulate during sulfonamide inhibition in the absence of purines, but the amine reverses the toxicity of sulfonamides by increasing the inhibition index about 3-fold. On the other hand, purines increase the inhibition index about 10-fold. These results suggest the possibility that sulfonamides first prevent the biosynthesis of the intermediate, a derivative of aminoimidazolecarboxamide, presumably at the stage of incorporation of the single carbon unit of the imidazole ring, and in the presence of the amine, inhibit its utilization at a still higher inhibition index.

The ability of cell suspensions of *Lactobacillus arabinosus* to utilize the amine is dependent upon the presence of glucose, phosphate and formate. While the first two may merely indicate a need for glycolysis under the experimental conditions, the formate requirement for increased utilization of the amine is as little as 1 $\gamma$ per ml. Higher concentrations of formate (1 mg/ml) increase the utilization of the amine from about 25% in the absence of formate to 80% of a concentration of 20 $\gamma$ of amine per cc. Other single carbon unit donors have some effect on the utilization of the amine (65).

The utilization of glycine in the biosynthesis of purines does not appear to involve the free amine as an intermediate, since isotopically labeled glycine in the presence of the amine is incorporated into purines but is not incorporated into the amine under the experimental conditions (66). Since inosinic acid appears to be formed before hypoxanthine on the basis of specific activity of the incorporated precursors (66), the amine ribotide is the likely conjugated form which may be the normal intermediate.

*Thymidine.* Assays for a factor which was isolated and identified as thymidine were based on the ability of thymidine to reverse the toxicity of x-methylfolic acid in a manner such that the inhibition index was increased 10-fold for *Leuconostoc mesenteroides 8293* as well as the ability of the factor to reverse the toxicity of either sulfonamides or 2,4-diamino-6,7-diphenylpteridine for *Lactobacillus arabinosus 17-5* (67, 11).

Since thymine did not exert such an effect, it was apparent that thymine represents a form of the pyrimidine which is not utilized by all organisms and that the biosynthesis of thymidine or a related conjugated form of thymine did not involve thymine per se as an intermediate.

Since the competitive inhibition of uracil by nitrouracil is prevented noncompetitively in the manner of a product by thymine for *Lactobacillus casei* which utilizes either thymine or thymidine, it appears that uracil or a related conjugated derivative serves as a precursor of a thymine derivative (11). While the contribution by the uracil moiety has not been fully determined, at least the ureido group appears to be involved on the basis of isotopic experiments (68, 69). The 5-methyl group of thymine has been shown to be derived from single carbon units such as formate. The competitive inhibition of thymine utilization by thiothymine (5-methyl-2-thiouracil) in *Lactobacillus casei* is reversed non-competitively by folic acid presumably by allowing direct synthesis of a conjugated form of thymine which can be formed from thymine but is inhibited by the analogue (70).

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# BIOSYNTHESIS OF PURINES AND PYRIMIDINES

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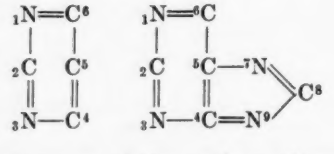
It has always been an interesting fact that the purines and pyrimidines, compounds which resemble each other structurally and which are so closely associated in the functions of the cell, have such different methods of synthesis and breakdown in the living organism. The products of pyrimidine catabolism are urea, $CO_2$, and water, whereas the purines are excreted as uric acid or allantoin. The most striking difference between these two groups of compounds, however, is the method the organism has in effecting their synthesis. As was shown some time ago, both compounds are rapidly synthesized *in vivo* from labeled ammonium salts (1) and other simple metabolic units.

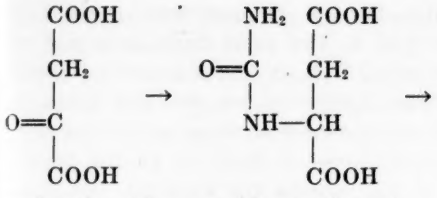


The precursors of the various nitrogen and carbon atoms of the purines are as follows: the ureide carbon atoms 2 and 8 from formate, carbon atom 6 from $CO_2$, and carbon atoms 4 and 5 and nitrogen atom 7 from glycine (2, 3).

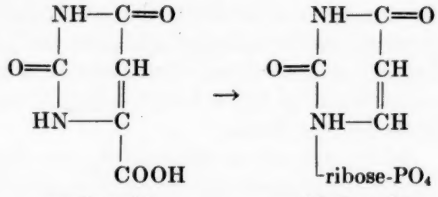
Of the above compounds, $CO_2$ is the only metabolic unit known to participate in the synthesis of the pyrimidine ring. It is known to enter position 2 (4) and possibly position 4 (5). The ureido-carbon of the pyrimidines is thus derived from $CO_2$ instead of formate as in the case of the purines. Neither formate nor glycine is apparently involved in biosynthesis of two pyrimidines, cytosine and uracil (4, 6), although formate, the $\alpha$ carbon of glycine and the $\beta$ carbon of serine have been reported as the precursor of the methyl group of thymine (7, 8). This latter result with thymine has not been repeated, however, in some laboratories (4, 9).

This difference in metabolic pathways of biosynthesis of these two groups of compounds suggested by the difference in precursors has been borne out by studies on the intermediates of purine and pyrimidine synthesis from the above elementary compounds.

Beginning with work on neurospora (10, 11), a series of compounds has been suggested as possible precursors of the pyrimidines. Among these are orotic acid, aminofumaric acid, aminofumaric acid diamide, and oxalacetic acid. Amino acids and compounds related to the tricarboxylic acid cycle were less effective in promoting growth of a uridine-requiring mutant. Ureidosuccinic acid has been postulated as an intermediate on the basis of experiments with rat tissue slices (12) and with *lactobaccillus bulgaricus* (13). Of the compounds listed, special attention will be given here to experiments with oxalacetate, ureidosuccinic and orotic acids. From the data gathered from various sources and indicated in table 1, oxalacetate seems to be the most likely elementary precursor of the pyrimidines. All other compounds such as aspartic acid (11, 14), members of the tricarboxylic acid cycle (11), pyruvic acid (11, 14) and lactic acid (15) are probably precursors by virtue of the fact that they may be converted into oxalacetate. Oxalacetate is used for pyrimidine synthesis by neurospora as well as by *lactobaccillus bulgaricus*, and by *E. coli* (13).




oxalacetic acid ureidosuccinic acid




orotic acid uridylic acid

Ureidosuccinate was sufficiently well used both by rat tissue slices (12) and by *lactobaccillus bulgaricus* (13) to indicate that it may have a place in a hypothetical scheme as shown above. Orotic acid has been extensively studied since the original finding of Loring and Pierce (10) and subsequent application by Mitchell and Houlahan (11) that it may be utilized for the growth of a uridine-requiring mutant of neurospora. Hammarsten, Bergström (16) and coworkers demonstrated that $N^{15}$ labeled orotic acid is readily incorporated into nucleic acid pyrimidines of the rat. The Philadelphia investigators have shown that orotic acid may be utilized for pyrimidine synthesis by yeast (15), by *lactobacillus bulgaricus* (13) and by *E. coli* (17). A step forward in the eventual enzymatic study of the reactions whereby orotic acid is converted into pyrimidines was made by Weed and Wilson (18, 19) who found that this reaction takes place using rat liver and spleen slices and homogenates. An indication of the mechanism of formation of the pyrimidine nucleotides from orotic acid is indicated from the work of Michelson, Frell and Mitchell (20) who have isolated orotic acid riboside from a natural source.

On the question of the biosynthesis of the purines, initial experiments in this laboratory (21) were mainly concerned with the establishment of the fact that in de novo synthesis of hypoxanthine in pigeon liver extracts, formate, $CO_2$ and glycine react in the stoichiometric proportions of 2:1:1 to form the complete purine ring. Advantage has been taken of this stoichiometry to determine the unknown nitrogen precursors of the hypoxanthine nitrogen atoms 1, 3 and 9. In these experiments carried out by Sonne and Lin (22), $C^{14}$ carboxyl-labeled glycine was incubated with suspected $N^{15}$ labeled precursors in pigeon liver extracts. Depending upon whether the suspected nitrogen precursor contributed none or some integral number of the 3 unknown nitrogen atoms of hypoxanthine, it was anticipated that each mole of newly formed hypoxanthine would contain 1 mole of $C^{14}$ labeled glycine and either 0, 1, 2 or 3 atoms of nitrogen from the tested nitrogen donor. With this technique it was found that in the pigeon liver extract, ammonium ions do not contribute to a significant extent to any of the 3 unknown nitrogen atoms. On the other hand, the amide nitrogen of glutamine contributes 2 atoms of N and the amino nitrogen of glutamic acid 1.2 atoms. The results with the $N^{15}$ labeled aspartic acid, although still inconclusive, indicate that it may contribute approximately 1 atom. It is possible that all these 3 organic forms of nitrogen contribute in a nonspecific manner to all 3 atoms of nitrogen in the purine ring (i.e., 1, 3 and 9). It is hoped, however, that after degradation of the purine ring, the results will show that some of the above compounds will contribute nitrogen atoms to specific positions of the purine ring in the same manner that the nitrogen of glycine is the specific precursor of nitrogen atom 7 of the ring.

TABLE 1. PYRIMIDINE SYNTHESIS BY VARIOUS ORGANISMS AND TISSUES

| Precursors | Neurospora | Yeast RNA | Rat Carcass | Rat Tissue Slices | Bacteria |
|---|---|---|---|---|---|
| $CO_2$ | | | +[1](4) | | |
| Formate | | −(6) | ±[2](4, 7) | | |
| Glycine | | −(6) | ±[2](4, 8, 9) | | |
| Serine | | | +[2](8) | | |
| Lactate | | +(15) | | | |
| Pyruvate | (+)(11) | | | (+)(14) | |
| Aspartate | (+)(11) | | | + (14) | +(12) |
| Oxalacetate | + (11) | | | + (12) | +(12) |
| Aminofumarate | + (11) | | | | |
| Aminofumaric acid di amide | + (11) | | | | |
| Ureidosuccinate | | | | + (12) | +(13) |
| Orotic acid | + (11) | +(15) | +(16) | + (18) | +(13, 17) |

[1] position 2 of uracil; [2] methyl group of thymine (DNA); (+) indicates slight response; + indicates strong response; − indicates compound probably is not a precursor.

The above results in the extract indicate the operation of transamination reactions in the biosynthesis of the purine ring and hence the involvement of pyridoxal phosphate, the coenzyme of the transaminating system.

During the course of investigating the metabolism of 4-amino-5-imidazolecarboxamide in pigeon liver with Dr. Martin P. Schulman and Mrs. Xenia Maehly (23, 24), a comparison was incidentally made of the incorporation of radioactive formate and radioactive glycine into inosinic acid. In contrast to previous experiments, inosinic acid was present in the medium during the incubation. In this experiment there was a surprising discrepancy in the amounts of formate and glycine that were incorporated into inosinic acid in comparison to the amounts one might have expected from considerations of de novo synthesis. The ratio of formate and glycine incorporated into inosinic acid was 166:1 instead of the expected 2:1. This indicated that formate was undergoing reactions in addition to those concerned with de novo synthesis. When the purine base of inosinic acid was degraded in such a manner as to compare the specific activities of carbon atoms 2 and 8 it was found that most of the extra formate radioactivity appeared in carbon atom 2. This same disproportionate incorporation of formate was observed in several experiments (*series 1*) but we also failed to observe this phenomenon in an equal number of experiments (*series 2*, table 2). In this second series of experiments the carboxyl carbon of glycine was incorporated into position 4 of the inosinic acid to about the same extent that formate was incorporated into positions 2 and 8. Inosinic acid was being synthesized in this latter series primarily but not exclusively by reactions of de novo synthesis.

In an attempt to reconcile the apparent discrepancies of these two series of experiments, it was observed that in *series 1* the de novo synthesis of purine from glycine was very small in comparison to that in *series 2*. This suggested the clue that the 'extra' reactions of formate at position 2 may be masked in some experiments by the occurrence of the de novo reactions in major proportions. In order to limit the extent of de novo reactions, one of the reactants of this system, $CO_2$, was omitted from the incubation medium. As shown in table 3, the omission of bicarbonate from the reaction medium resulted in a severe reduction in the synthesis of inosinic acid from glycine and a large decrease in the incorporation of formate into position 8. Although the amount of formate incorporated into position 2 was less in the absence of bicarbonate than in its presence, the reduction of radioactivity incorporation into this position was substantially less than that in positions 4 and 8. It is particularly noteworthy that in the experiment reported in table 3, glycine and formate are utilized for inosinic acid in proportions indicating primarily de novo synthesis of purine when bicarbonate is present. In its absence the total incorporation of formate into positions 2 plus 8 as compared to the utilization of glycine was 8:1. Moreover, the ratio of incorporation of formate into position 2 as compared to position 8 was 3.6:1. These results indicate that by omission of bicarbonate from the reaction medium, the extent of the de novo reactions is severely reduced making evident the existence of the extra or 'exchange' reaction of formate. In the experiments reported in table 2, bicarbonate was present in the vessels of both series of experiments. It is therefore probable that in the experiments of *series 1*, the de novo reactions were reduced by the fortuitous absence of some

TABLE 2. PURINE SYNTHESIS IN THE PRESENCE OF BICARBONATE AND INOSINIC ACID

| Series | Micromoles radioactive substrates incorporated per millimole inosinate: Formate positions 2 | 8 | Glycine position 4 | Relative incorporation of radioactive substrates: Position no. 2 | 8 | 4 |
|---|---|---|---|---|---|---|
| 1 | 11.6 | 0.54 | 0.07 | 159 | 7 | 1 |
| 2 | 21.0 | 14.6 | 16.3 | 1.30 | 0.9 | 1 |

TABLE 3. EFFECT OF BICARBONATE ON INCORPORATION OF RADIOACTIVE SUBSTRATES INTO INOSINIC ACID

| Vessel | Bicarbonate present or absent | Micromoles radioactive substrate incorporated per millimole of inosinic acid: Glycine position 3 | Formate positions 2 | 8 | Relative incorporation of radioactive substrates into Positions 4 | 2 | 8 |
|---|---|---|---|---|---|---|---|
| 1 | Absent | 0.54 | 3.48 | 0.98 | 1 | 6.5 | 1.8 |
| 2 | Present | 16.3 | 21.4 | 14.9 | 1 | 1.3 | 0.9 |

TABLE 4. EFFECT OF HYPOXANTHINE, INOSINE AND INOSINIC ACID ON INCORPORATION OF RADIOACTIVE SUBSTRATES INTO PURINES[1]

| Vessel | Additions | Micromoles radioactive substrate incorporated into total 'hypoxanthine compounds' of each vessel: Glycine position 4 | Formate positions 2 | 8 | Ratio of formate incorporated into positions 2 and 8 of inosinic acid |
|---|---|---|---|---|---|
| 1, 2 | None | 0.012 | 0.014 | 0.010 | 1.5 |
| 3, 4 | Hypoxanthine | 0.011 | 0.012 | 0.008 | 1.5 |
| 5, 6 | Inosine | 0.010 | 0.013 | 0.008 | 1.5 |
| 7, 8 | Inosinate | 0.002 | 0.020 | 0.003 | 6.6 |

[1] Bicarbonate omitted.

other factor required for the synthesis, either of enzyme or coenzyme.

The participation of inosinic acid in the so-called enzymatic 'exchange' reactions of formate with the carbon atom at position 2 is demonstrated in table 4. In the absence of compounds of inosinic acid, however, the incorporation of glycine into position 4 or formate into position 8 is severely depressed whereas the incorporation of formate into position 2 is doubled. The ratio of formate radioactivity in position 2 as compared to position 8 was approximately 6.6:1, indicating the operation of the 'exchange' reaction of formate with the carbon atom of position 2 of inosinic acid.

In view of the evidence which has accumulated indicating that a folic acid derivative is involved in the synthesis of the purine ring, we tried the effect of two compounds, folic acid and the

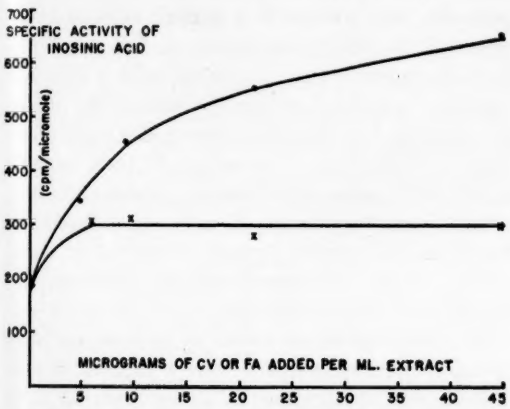



Fig. 1. Effect of citrovorum factor and folic acid on incorporation of radioactive formate into inosinic acid. ● = citrovorum factor; × = folic acid.

Table 5. Effect of citrovorum factor on incorporation of radioactive substrates into inosinic acid[1]

| Vessel | Citrovorum factor added or omitted | Micromoles radioactive substrate incorporated per millimole inosinic acid | | | Stimulation of incorporation of radioactive substrates into purine position by citrovorum factor (micromoles) | | |
|---|---|---|---|---|---|---|---|
| | | Glycine position | Formate positions | | | | |
| | | 4 | 2 | 8 | 4 | 2 | 8 |
| 1 | Omitted | 0.22 | 2.35 | 0.38 | | | |
| 2 | Added | 0.75 | 5.07 | 0.76 | 0.5 | 2.7 | 0.4 |

[1] Bicarbonate omitted.

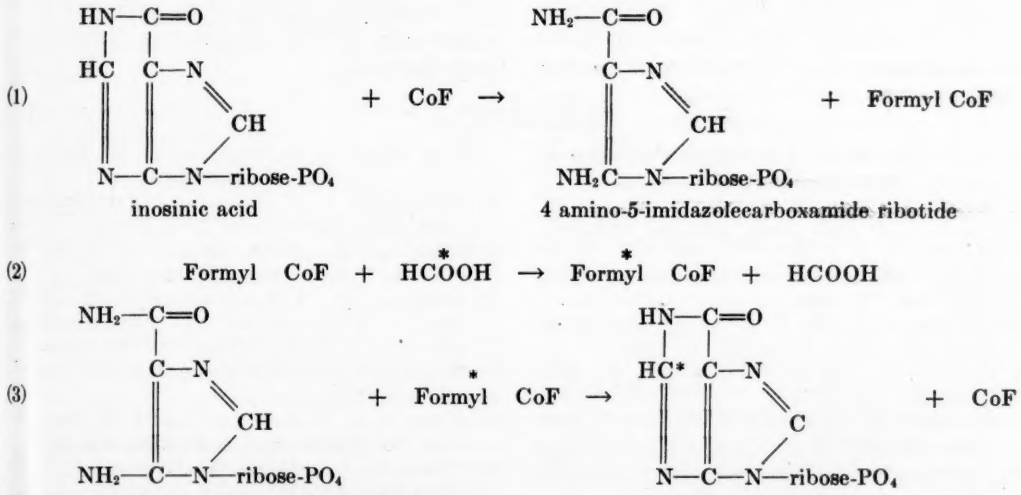



Fig. 2. Possible reactions of formate and inosinic acid. CoF (coenzyme F) indicates 5, 6, 7, 8 tetrahydrofolic acid. Formyl CoF (citrovorum factor) indicates $N^5$ formyl 5, 6, 7, 8 tetrahydrofolic acid. * designates radioactive carbon.

other than formate and glycine, these substances are utilized for purine reactions approximately in proportions expected from de novo synthesis. The addition of hypoxanthine or inosine to the incubating medium does not materially change the ratio of this incorporation. In the presence citrovorum factor, on the incorporation of formate into inosinic acid. The citrovorum factor used in these experiments was leucovorin, which is reported to be $N^5$ formyl 5, 6, 7, 8 tetrahydrofolic acid (25, 26). Both compounds when added in small amounts to pigeon liver extracts in-

creased the incorporation of radioactive formate into inosinic acid. As shown in figure 1, there was an initial response to folic acid when added in a concentration up to 5γ/ml of extract. Thereafter, however, there was no further stimulation at higher concentrations of vitamin. In the case of leucovorin, however, there was a continued response to increased levels of this compound until at concentrations of 45 γ/ml the amount of radioactive formate incorporated into inosinic acid was approximately 3 times that of the control without added vitamin. These experiments were carried out in the presence of bicarbonate, so that the stimulatory effects of the citrovorum factor noted could have been on both the de novo reactions and on the 'exchange' reaction of formate.

It was of particular interest to determine whether the citrovorum factor could act by stimulating specifically the 'exchange' reaction of formate with the carbon atom at the 2 position of inosinic acid. An experiment was therefore carried out with pigeon liver extracts in which bicarbonate was excluded from the medium, an experimental state where the 'exchange' reaction of formate is primarily evident. As shown in table 5, the addition of the citrovorum factor resulted primarily in a stimulation of the 'exchange' reaction of formate with the carbon atom at position 2 of inosinic acid, although there was a small increase in the de novo synthetic reactions as indicated by an increased incorporation of glycine into the purine ring.

With the above information, a scheme has been suggested to explain the incorporation of formate primarily into position 2 of inosinic acid and the effect of the citrovorum factor on this reaction. In this scheme (figure 2) inosinic acid is cleaved yielding 4-amino-5-imidazolecarboxamide ribotide and the formylated coenzyme F (citrovorum factor). This formyl coenzyme F then equilibrates with radioactive formate, yielding radioactive formyl coenzyme F and non-radioactive formate. Radioactive formyl coenzyme F then reacts with 4-amino-5-imidazolecarboxamide ribotide to form inosinic acid-2-$C^{14}$. The proposed role of the citrovorum factor as an acceptor and donor of formyl groups is thus a parallel of the known role of coenzyme A as an acceptor and donor of acetyl groups.

These experiments in conjunction with those using radio-active 4-amino-5-imidazolecarboxamide are suggestive of the important role which the hypothetical compound, 4-amino-5-imidazolecarboxamide ribotide, may play in purine synthetic reactions.

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# MECHANISMS INVOLVED IN THE BIOSYNTHESIS OF PURINES[1]

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IN STUDYING the various reactions which participate in the biosynthesis of the purines it is necessary immediately to recognize that we know little about the intermediate compounds involved. In fact in some instances, we do not know the exact substrates and in certain respects we are not even certain of the products. In order to study the actual enzymes and cofactors involved in this biosynthesis, it becomes necessary to elucidate the pathway by isolation of intermediate compounds.

When we began our studies on hypoxanthine synthesis in pigeon liver preparations, we considered, together with others, that free purines were being synthesized (1–4). It has since been shown that at least in the case of hypoxanthine the purine rings are completed only after introduction of ribose and phosphate moieties (5). A working picture of purine biosynthesis is shown in figure 1. Thus glycine, an ammonia derivative (5a, 5b) and a ribose phosphate compound (perhaps ribose-1,5-diphosphate) (5c) may form a compound of the type shown at I. Formylation to form II may be followed by introduction of ammonia derivatives and a $CO_2$ derivative and by ring closure to 5-$NH_2$-4-imidazolecarboxamide-5′-phosphoribotide (III)[2]. Finally formylation and closure to yield inosinic acid (IV) occurs. Present evidence suggests that the free base of 4-amino-5-imidazolecarboxamide be excluded since it is generally agreed to be derived from the true intermediate (4–7). Certainly other equally tenable pathways may exist. Thus the imidazole ring might close before the $CO_2$ portion of the eventual purine molecule is incorporated. I have arbitrarily assigned the structures indicated at I and II as types of compounds which could be involved, since these more immediate precursors of imidazole ribotide are still hypothetical. Buchanan and coworkers (8) appear to have ruled out a number of possible compounds.

Space does not permit discussion of evidence for the present scheme. It represents the combined efforts of several groups of workers (1, 4–14). Actually none of these intermediates has been clearly identified, and much of the scheme is based on what seems to be logical reasoning.

### STUDIES ON SULFADIAZINE-INHIBITED ESCHERICHIA COLI

In an effort to accumulate and characterize some of the intermediate compounds involved in purine biosynthesis, we have turned our attention to sulfonamide-inhibited *Escherichia coli* cells. The inhibition of the action of or the synthesis of cofactors should result in the accumulation of metabolites, and lead to greater insight into the mechanism of action of the cofactors. Shive and coworkers (11) have identified the amine isolated by Stetten and Fox (15) from such cultures as 4-amino-5-imidazolecarboxamide. It has been considered that the accumulation of the carboxamide in the sulfonamide-inhibited cultures results from the inhibition of the ring closure at carbon 2 of the purine because of the deficiencies of cofactors whose exact nature is still uncertain (6) but which seem to be derived from para-aminobenzoic acid. Thus far only the free carboxamide has been shown to occur. Since, however, the inhibition is presumably at the point of closure of 5-$NH_2$-4-imidazolecarboxamide-5′-phosphoribotide to form the purine nucleotide (figure 1, III–IV) it would be expected that the ribotide (III) and the riboside of imidazolecarboxamide would accumulate as well as the free base.

[1] The studies reported here were aided by grants from The National Foundation for Infantile Paralysis, and the Elisabeth Severance Prentiss Foundation.

[2] In this discussion the general terms carboxamide compounds, imidazole compounds, and diazotizable amine fraction refer to 4-amino-5-imidazole-carboxamide and its glycoside derivatives. Where the imidazole compound derived by closure of carbon 8 of the eventual purine is mentioned, compounds of the type shown at III in figure 1 are intended. Total amine determination refers to the diazotizable, non-acetylatable (aromatic) amine fractions and would include such compounds as III of Fig. 1 as well as 4-$NH_2$-5-imidazolecarboxamide.

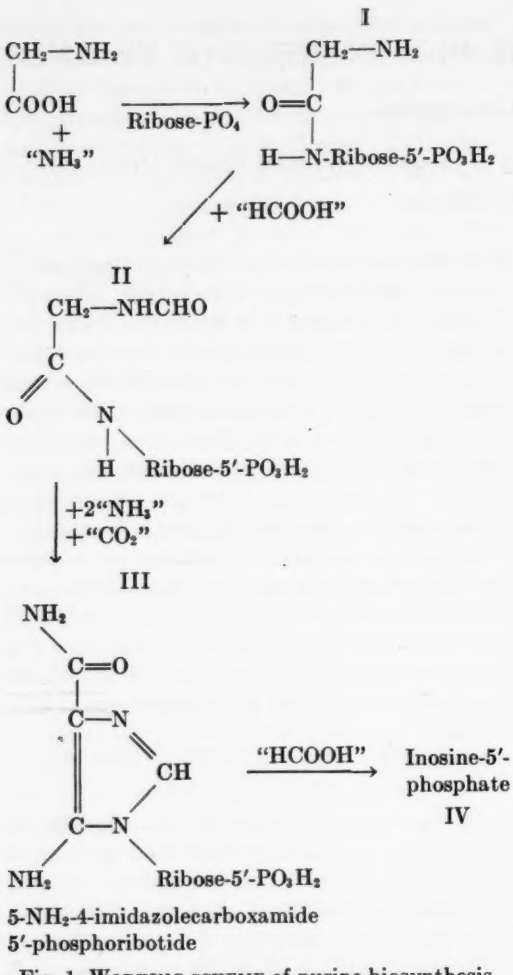


Fig. 1. WORKING SCHEME of purine biosynthesis.

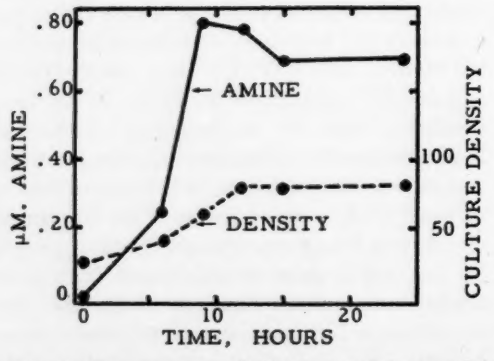


Fig. 2. RELATIONSHIP between total (aromatic) amine production and time after inoculation in *E. coli* cultures inhibited by sulfadiazine. 0.4 ml of a 16 hour normal culture grown in glucose-$NH_4Cl$-salts medium was inoculated into 10 ml of fresh culture medium containing sulfadiazine and glycine. Culture density refers to Klett readings.

Another question arises as to why the closure of the pyrimidine ring (carbon 2) is apparently affected by sulfonamide inhibition and not the closure of the imidazole ring (carbon 8 of the purine). It is known that carbon 8 as well as carbon 2 is derived from formate. The mechanism of introduction of carbons 2 and 8 would be expected to be similar. We have considered the possibility that the incorporation of both carbons 2 and 8 is inhibited by sulfonamide and that both reactions require the same cofactors. It would appear that this might occur and that the detection of the imidazole precursor would be limited only by an ignorance of its nature. If this were true, we might theorize that the cofactor would be more easily dissociated from the $C_2$ closure enzyme than from the $C_8$ enzyme. Therefore closure at carbon 8 would occur at a concentration of cofactor which would be too low for carbon 2 closure. It could not be the reverse since otherwise the imidazolecarboxamide would not accumulate at all, and only the imidazole precursor would occur. In fact there is suggestive evidence that as the cofactor deficiency increases, the imidazole precursor may appear.

With these concepts in mind, we have investigated *E. coli* cultures inhibited by sulfonamide. There is not space to relate the requirements of the system in detail. One important factor is the quantity of inoculum. These studies have been carried out with *E. coli*, strain B, employing glucose, $NH_4Cl$ and salts as the medium in the presence of sulfadiazine and glycine. The cultures have been grown aerobically at 37° without agitation.

In figure 2 is shown the relationship between the total amine production per 10 ml, of sulfadiazine-inhibited *E. coli* culture and the time after inoculation. A characteristic maximum at about 10–12 hours is observed followed by a fall and then a plateau. It will be observed that the amine production is most rapid during the period of increasing culture density. The culture density is about ½ that observed in normal growth. Larger inocula produce lower maxima earlier in time and lower inocula produce maxima which are less striking. While these studies were in progress, Bergmann and coworkers (16) published such an amine production curve, but their inoculating culture was smaller and the maximum was less prominent.

From the results in figure 2 it may be considered that in the early stages of the inhibition

the amine is being produced more rapidly than it is apparently being converted to purine, followed by a period when the disappearance exceeds the synthesis, and followed finally by a plateau in which the amine remains constant. From this time curve we concluded that the closure of the imidazole ring occurs only up to the time of the occurrence of the maximum. If we accept the premise that sulfonamide inhibits the closure of carbon 8 as well as that of carbon 2, then as the cofactor deficiency becomes more pronounced with time, closure of carbon 8 will be stopped, no carboxamide will occur, and the carboxamide precursor should accumulate.

In order to study this we set up the experiment shown in figure 3. In one set of culture tubes, glycine was added at the time of the inoculation (0 hours). In another, glycine was added at 8 hours, and in another glycine was added at 15 hours. All tubes contained sulfadiazine. Measurement of total imidazole amine was made at 8, 10, 15 and 17 hours as indicated. It will be observed that without glycine the amine production is constant and relatively slow; this suggests that glycine formation by these inhibited cultures is a limiting reaction. When glycine is added at time zero, the amine production is more rapid, increasing sharply at about 7–8 hours and then passing through the maximum value as estimated by the dashed lines. When glycine is not added until 8 hours the rise in amine production is exactly parallel to that observed when glycine is added at time zero. On the other hand addition of glycine at 15 hours does not cause the sharp rise in amine production observed at the earlier times.

We may analyze this situation further by a consideration of the following reactions:

$$\text{glycine, etc.} \xrightarrow{(1)} \text{“X”} \xrightarrow{(2)} \text{Imidazole compound}$$

in which (*1*) represents a series of reactions forming an imidazole precursor and *reaction* (*2*) is a system effecting closure of this precursor to form the imidazole compound. We may conclude that glycine is not converted to imidazolecarboxamide amine after 15 hours and presumably *reaction* (*2*) has been completely inhibited. In addition, we may tentatively conclude that *reaction* (*1*) is limiting in the absence of glycine.

In order to study this problem further and to determine whether compound “X” might indeed be accumulating when glycine is added to sulfadiazine-inhibited cultures at 15 hours, carboxyl-labeled glycine was employed in the same type of experiment as shown in figure 3. $C^{14}$ glycine was added to each of two sulfadiazine-inhibited *Escherichia coli* cultures, in one case 8 hours after inoculation and in the other 15 hours after inoculation. Incubation with labeled glycine was carried out for 2 hours. The culture media from the washed cells of both incubations were lyophilized and aliquots of each were subjected to paper chromatography employing a butanol, diethylene glycol, $H_2O$, $NH_3$ mixture as the solvent (17), and the paper chromatograms analyzed for $C^{14}$ activity (5) The results of these chromatograms are shown together in figure 4. Consider the compounds occurring after the earlier incubation. *Peak C* corresponds in $R_F$ to the free 4-$NH_2$-5-imidazolecarboxamide ($R_F$ 0.50), and shows a strong diazotization reaction. *Peak B* corresponds to glycine. The slowest moving component (A) has been tentatively identified as a phosphopentotide of imidazolecarboxamide. Its $R_F$ corresponds to that of the purine ribotides. On extraction this compound showed a slight but definite diazotization reaction indicating an aromatic amine as in the carboxamide. After dilute acid hydrolysis the major portion of the $C^{14}$ activity migrated

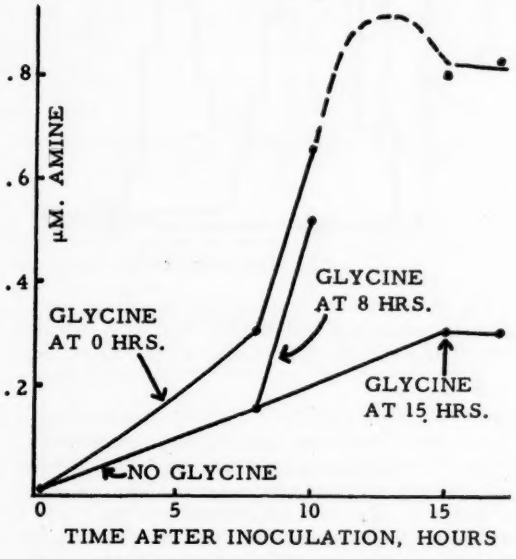


Fig. 3. Effect of glycine on amine production when added at different times after inoculation to sulfadiazine-inhibited cultures. Glycine was added to cultures as indicated (see text).

to the position of the carboxamide base ($R_F$ 0.5). From the scheme shown in figure 1 the accumulation of the pentotide would be expected. It is clear from these experiments that the imidazolecarboxamide fraction and the pentotide fraction are not formed when radioactive glycine is added at 15 hours. This confirms the

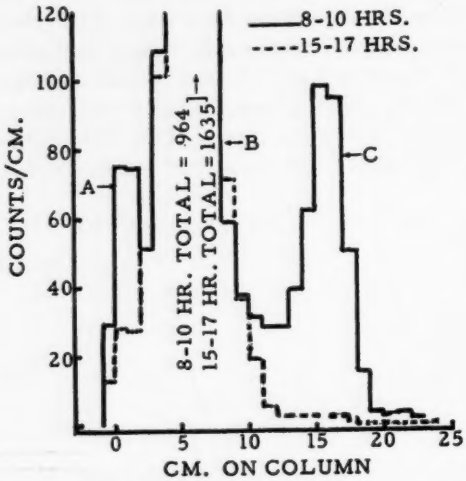


Fig. 4. Paper chromatograms of culture media after incubation of sulfadiazine-inhibited cultures with $CH_2C^{14}OOH$. $C^{14}$ glycine added to sulfadiazine-inhibited cultures 8 hr after inoculation of the culture (unbroken line). $C^{14}$ glycine added at 15 hr (broken line). Incubation with labeled glycine was 2 hr in each instance. Figures under *Fraction B* refer to total counts between 4 and 8 cm on chromatograms. Sulfadiazine was found at $R_F$ 0.76 (24 cm) 2.9 $\mu$M$C^{14}$ glycine (24,000 counts/$\mu$M) added to 10 ml. culture. Data are for 1 ml. of culture medium.

Table 1. Distribution of activity after incubation of carboxyl-labeled glycine with sulfadiazine-inhibited *Escherichia coli*

| | Counts/min/ml. of culture | | | |
|---|---|---|---|---|
| $C^{14}$ glycine added at | Total | Glycine | Imidazole Fractions | "X" |
| 8 hours | 1,890 | 1,370 (−X?) | 504 | ? |
| 15 hours | 2,040 | 1,590 | 0 | 440 |

See figure 4.

quantitative data shown in figure 3. Since the total activity of the culture medium was approximately the same at 17 hours and at 10 hours, the question arises whether the $C^{14}$ glycine has accumulated in a precursor of the imidazole compounds when the incubation is conducted at 15 hours.

Since no $C^{14}$ imidazolecarboxamide compound is formed when glycine is added 15 hours after inoculation of the *E. coli* culture, another aliquot of this reaction mixture was chromatographed on paper with 60% propanol in an attempt to separate "X" from the glycine. Two fractions, one of which was glycine, resulted. The data on the "X" formation in one experiment are summarized in table 1. These data are estimated from paper chromatograms. Notice that the counts in "X" formed from glycine between the 15th and 17th hour account quite well for the diminished imidazolecarboxamide formation. Since little activity is found in the cells in either culture it appears that "X" accumulates mainly when carboxamide does not. When a more concentrated suspension of cells grown for 15 hours in sulfadiazine is employed a larger proportion of compound "X" is formed from glycine. We have estimated that when glycine was added at 15 hours, about 50 $\mu$M of the "X" compound were formed per 2 hours by the inhibited cells from 1 liter of culture. This agrees well with about 40 $\mu$M of imidazolecarboxamide formed per 2 hours during the period 8–10 hours.

It is not known with certainty whether compound "X" is actually a precursor of imidazolecarboxamide compounds. Clearly, however, the pathway from glycine to the "X" fraction is rather direct. Thus at 15–17 hours almost all of the activity from $C^{14}$ glycine is found as "X". From control chromatograms X is not serine. Furthermore serine would not be expected to be formed in the presence of sulfonamide. We are now in the process of separating this compound from glycine. It will be necessary to add "X" back to the 8 hour culture in order to test whether it can form carboxamide in the absence of glycine.

It is of interest to speculate as to the reason that other bacteria whose growth is inhibited by sulfonamide do not accumulate the carboxamide compounds. If in these cases the cofactors involved dissociated from the carbon 8 closure enzyme more easily than from the carbon 2 closure enzyme, only the imidazole compound precursor should occur.

In order to investigate the nature of the imidazolecarboxamide fraction more fully, we have employed a larger volume of culture. After growth in the presence of sulfadiazine, glycine was added at 7 hours and the incubation continued 2 hours. The culture medium was lyophilized, placed on large sheets of filter paper, and chromatographed with propanol-water solvent. The aromatic amino group of imidazolecarbox-

amide was detected by spraying with diazotization reagents.

Two diazotizable amines were detected in addition to the sulfadiazine. In this run no imidazole pentotide was found. One fraction, I-1, which is present in lesser quantity than I-2 has been divided by fractionation with other solvents into 4 distinct ultraviolet-absorbing compounds, 2 of which show diazotization reactions and none of which seems to correspond to the free imidazolecarboxamide. They do not give the orcinol or the Stumpf desoxypentose tests. These compounds are being investigated.

Fraction I-2 on rechromatographing with several solvents has remained as a single component. This has been assigned the tentative structure 5-$NH_2$-4-imidazolecarboxamide-N-pentoside.

The evidence for this is shown in figures 5 and 6 and in table 2. It will be observed (fig. 5) that the absorption spectrum of the pentoside is quite similar to that of the free carboxamide. In table 2 are shown the relative molar values of this pentoside in terms of its ultraviolet maximum, its diazotizable amine value and its pentose content. The data indicate ratios of close to 1:1:1. It is to be noted that the first two figures are based on the values for the free base as a standard and these are not necessarily the same as those of the pentoside.

Figure 6 shows that when the pentoside is hydrolyzed with dilute acid, it liberates the free carboxamide as is shown by chromatographing the compound before and after hydrolysis and comparing the $R_F$ values with authentic carboxamide. The liberated carboxamide gives the typical diazotizable amine reaction.

The aminoimidazolecarboxamide pentoside exhibits a rather characteristic instability. Thus when allowed to stand on filter paper after chromatography the compound forms a red derivative. This behavior has not been observed with the free base under similar conditions. It may be of interest to note that various workers have reported the occurrence of pigments in systems in which purine synthesis is inhibited (18, 19).

It is not definitely known whether this pentoside is an intermediate in the reaction. It is present in considerably greater concentration than the other diazotizable amines. Perhaps it represents a degradation product from the ribotide by phosphatase action. It is quite conceivable that a free base (not the carboxamide) is converted to a ribotide directly by reaction with ribose-1,5-diphosphate, as can be inferred from the work of Williams and Buchanan (5c). Whatever the role of the imidazolecarboxamide pentoside, its isolation and characterization strengthens the suggestion that the carboxamide as such is not the intermediate compound and that closure of carbon 2 of the purine occurs at a ribose derivative level.

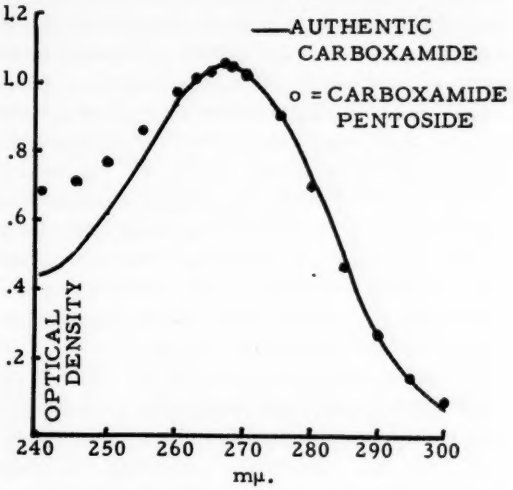


Fig. 5. Absorption spectrum of imidazolecarboxamide pentoside compared to the synthetic carboxamide at pH 7.

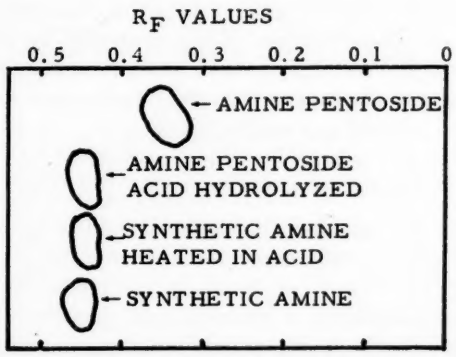


Fig. 6. Chromatographic evidence of formation of 4-$NH_2$-5-imidazolecarboxamide by dilute acid hydrolysis of the imidazolecarboxamide pentoside.

Table 2. Relative molar values of 5-$NH_2$-4-imidazolecarboxamide pentoside

| $\epsilon_{267m\mu}$ | Diazotizable amine | Pentose |
|---|---|---|
| 1.17[1] | 1.00[1] | 1.00 |

[1] These values employ the free base as a standard using $\epsilon_{267}$ $1.27 \times 10^4$.

It is important that a pentoside, presumably a riboside, accumulates whereas as yet we have not been able to find evidence for a desoxypentose fraction. One might believe that the pathway of purine biosynthesis would involve both ribose and desoxyribose derivatives. Yet it is possible that desoxypentose derivatives are formed from pentose compounds at a higher level of complexity of the nucleic acid precursors. It is of some interest that little, if any, of the free carboxamide has been found when the experiment was carried out as described. This may suggest that the free carboxamide is formed only by degradation later in the incubation. In addition, it may be added that the earlier studies (11, 15) of this compound employed procedures which would have hydrolyzed the imidazole-ribose linkage.

It should be mentioned that Ben-Ishai *et al.* (20) recently presented preliminary evidence for the formation of a glycoside fraction of imidazolecarboxamide, apparantly consisting of both desoxyribose and pentose, when the free base of the carboxamide is incubated with *E. coli*. The absorption maximum obtained by these workers was somewhat different than that of the free base. The present studies extend this observation in that the formation of the pentoside by the synthesis from glycine and other precursors of purine is shown and in that the compound has been isolated in a purified state.

### FORMATE ACTIVATION AND RING CLOSURE

So far the products which accumulate in the presence of inhibitors of cofactors involved in the ring closures of the purine precursors have been considered. I would like to consider now some studies in pigeon liver preparations on the mechanism of activation of formic acid and the closure of the rings. We have reported that an extract of acetone powder of pigeon liver is able to fix $C^{14}$-formate anaerobically into inosinic acid (IMP-5) and that this requires ATP, glutamine, a source of ribose-phosphate, bicarbonate, glycine, and finally boiled extract (5a). The boiled extract could be replaced in part by citrovorum factor, and biotin had a small effect. At the same time Buchanan and his coworkers found that the $C^{14}$-formate was able to exchange with carbon 2 of inosinic acid independently of total synthesis and that this reaction required citrovorum factor. It is now apparent that in part we also were studying this exchange reaction. But, in addition, incorporation into carbon 8 was occurring, and apparently this too represents an exchange at least in part.

*Homocysteine Requirement and Formate and Formaldehyde Incorporation.* Efforts to clarify the mechanism involved in the formate fixation into purine were favored by a fortunate coincidence. It had previously been reported that both formate and formaldehyde were incorporated into carbons 2 and 8 of the purine fraction without having to pass through each other (5a). Thus there had to be a common intermediate for these two compounds and we depicted it in the following manner: Formate may react with some carrier (perhaps an amino compound) to form the N-formyl derivative while $CH_2O$ may react with this carrier to form the N-hydroxymethyl compound, and these compounds would be expected to be in equilibrium with each other. Later Paul Berg in our department, studying methionine synthesis from homocysteine and $C^{14}$-formate in pigeon liver preparations, also observed that both formaldehyde and formate were converted to the methyl carbon of methionine, again without obligatorily passing through one another. We both have felt that the conversion of formate and formaldehyde to methyl carbon and to purine carbons 2 and 8 had something in common and that this scheme was involved in both instances, the methionine being formed at the hydroxymethyl level and purine from the formyl level. Berg has made the important observation that in the presence of homocysteine a considerable amount of $C^{14}$-formate was fixed into compounds which were not methionine and the possibility was considered that this was the purine fraction. In analyzing his reaction system for activity in purine he observed that in the presence of homocysteine the activity in the carbons 2 and 8 of the total hypoxanthine fractions was much greater than in the absence of homocysteine. To follow this interesting lead, we studied the effect in acetone powder extracts under conditions which I have just described. We have found that homocysteine is apparently specific in its stimulation of the incorporation of formate into inosinic acid since other sulfhydryl compounds were without effect. Since the possibility exists that this effect is concerned with reactions leading up to the imidazolecarboxamide ribotide, the presumed precursor of inosinic acid, homocysteine was tested in a system in which exchange of the inosinate carbon 2 with $C^{14}$-formate (21) is super-

imposed on the *de novo* synthesis. The reaction was carried out anaerobically as before, with an extract of acetone powder dialyzed 2 hours in order to remove substrate preferentially and possibly still to maintain the cofactors. We are not sure this has been accomplished. Table 3 shows the results of a typical experiment. To this system were added inosinic acid, ATP, phosphoglycerate, $C^{14}$-formate, buffers and the additions as shown. The maximum incorporation value represents a minimum of approximately 0.2 $\mu$M of $C^{14}$-formate per 20 minutes for the extract from 40 mg. of acetone powder. Homocysteine has a specific stimulatory effect not shown by other sulfhydryl compounds or reducing substances tested. Homocysteine increases the incorporation of formate into both the 2 and 8 carbons, but quantitatively the effect on the incorporation into the number 2 carbon is as much as 5 times greater.

The glutathione effect is apparently a protection of the trace of homocysteine present since in most experiments glutathione has no effect. That this homocysteine effect may be on the activation of formate in general is derived from the findings of Berg (22), that addition of homocysteine to pigeon liver increases serine synthesis and that other reducing compounds are ineffective in increasing total formate fixation.

*ATP Requirement.* Our early experiments have shown a definite need for ATP in the incorporation of $C^{14}$-formate (5a). To study this more specifically we have employed the system described above for the homocysteine experiments. The results are shown in table 4. A minimum of approximately 0.6 $\mu$M of $C^{14}$-formate was incorporated in the undialyzed complete system. It will be observed that in both the dialyzed and undialyzed systems the removal of ATP and 3-phosphoglycerate together greatly diminished the $C^{14}$-formate incorporation and that apparently phosphoglycerate is necessary for regeneration of the low level of ATP. In both dialyzed and undialyzed extracts the ratios of activity with and without ATP are almost identical. It is not likely that ATP is serving only as a source of IMP-5 since addition of much greater levels of inosinate does not diminish the requirement for ATP. It cannot yet be stated that these studies clearly differentiate between the ATP requirement for the *de novo* synthesis of the nucleotide skeleton and the requirement for activation of formate.

Finally, a tentative scheme for the activation of the one carbon unit may be considered as shown in figure 7. This is suggested only as a working mechanism since the evidence for its formulation is circumstantial. Here homocysteine (RSH) is suggested as the carrier described

TABLE 3. EFFECT OF HOMOCYSTEINE ON $C^{14}$ FORMATE UPTAKE INTO PURINE

| Additions | Counts incorporated into carbons 2 and 8 |
|---|---|
| None | 1240 |
| Homocysteine | 2980 |
| Glutathione | 1900 |
| Ascorbic acid | 1240 |
| Cysteine | 840 |

Reaction system: 0.4 ml. dialyzed extract (1:10 extract of acetone powder), 0.85 $\mu$M Na-inosinate, 2.5 $\mu$M ATP, 15 $\mu$M Na-phosphoglycerate, 80 $\mu$M $KHCO_3$, 8 $\mu$M $MgCl_2$, 8 $\mu$M $C^{14}$ formate. The additions in the table were 17 $\mu$M. Final volume 1.15 ml. Gas phase 95% $N_2$ − 5% $CO_2$. Reaction time 20 min. Temp. 38°.

TABLE 4. EFFECT OF ATP ON $C^{14}$-FORMATE UPTAKE INTO PURINE

| Extract | Counts in Purine (Carbons 2 and 8) Complete | Minus ATP and Phosphoglycerate | Minus Phosphoglycerate |
|---|---|---|---|
| Undialyzed + boiled extract | 6490 (1.0) | 527 (.081) | 3880 (.060) |
| Dialyzed | 673 (1.0) | 61 (.091) | 417 (.062) |

The system is similar to that used in table 3 but with 2$\mu$M of IMP-5. Reaction time 20 min. Figures in parentheses correspond to the relative radioactivity in each instance assigning a value of 1.0 to the complete system.

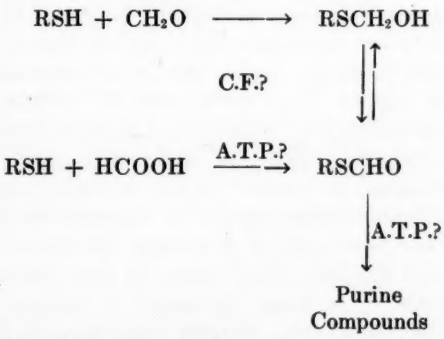


Fig. 7. SUGGESTED scheme for activation of formate and formaldehyde in synthesis of purine.

previously as being possibly an amino compound. According to this suggested scheme, formate would react with homocysteine perhaps through the mediation of citrovorum factor to form S-formylhomocysteine while $CH_2O$ would form S-hydroxymethylhomocysteine. The two com-

pounds are regarded as being in equilibrium by an oxidation-reduction system. Methionine and serine may be considered to be derived from the hydroxymethyl level. It is not clear how ATP should be included in this scheme. From the analogy of the requirement of an energy-rich phosphate donor in the activation of acetate to acetyl-coenzyme A (23), it might be expected that ATP would participate in the activation of formate to the S-formylhomocysteine level. However, Berg has found that ATP actually inhibits the incorporation of formate into methionine in the whole extract. It is quite possible that as the system is broken into its component parts the ATP may be shown to be involved in forming such a compound as formylhomocysteine. In addition it is not possible to state clearly whether ATP is involved in the system activating formate or in the activation of the purine precursor acting as the formate acceptor or in both. These problems are being attacked by fractionation studies.

Bergmann and coworkers (16) recently have provided data which they interpret as evidence for the conversion of the methyl carbon of methionine into carbon 2 of the purines in *E. coli* partially inhibited by sulfadiazine. It is, however, conceivable that formylhomocysteine may be derived from methionine and act as a carrier. In the pigeon liver system methionine added as a pool does not dilute the radioactivity incorporated into inosinic acid from $C^{14}$-formate.

*Citrovorum Factor Involvement.* Citrovorum factor or a derivative is somehow concerned in the purine synthesizing system. It has been implicated in the closure of carbon 2 of the purine (24). Our studies have shown that $C^{14}$-formate incorporation into inosinic acid is stimulated by citrovorum factor (5a) and more specifically Buchanan has shown that the carbon 2 exchange of inosinate and formate is activated by citrovorum factor (8). It is possible that citrovorum factor acts as a formyl carrier in these reactions. However, it seems necessary to include the observation that formate and formaldehyde apparently form a common intermediate in their pathway into carbons 2 and 8 of inosinic acid. Thus if the citrovorum factor is a formyl carrier and the only carrier in the system, it is to be expected that it would need to be capable of also carrying a hydroxymethyl group and of intraconversion of the —CHO and —$CH_2OH$ groups. The recent studies of Elwyn, Weissbach and Sprinson (25) suggesting that the $\beta$-carbon of serine may be converted to methyl groups without exchange of hydrogen possibly indicate a carrier at the —$CH_2OH$ level. The implication of homocysteine as an additional component of the one-carbon activating system might suggest a scheme such as presented in figure 7. Where and how citrovorum factor would fit into such a scheme remains for future studies.

## SUMMARY

Some of the problems involved in the biosynthesis of purines have been reviewed. We have assumed that the effect of sulfadiazine in *Escherichia coli* is not only to prevent closure of carbon 2 but also of carbon 8 of the purine depending on the relative cofactor deficiency. Such a concept has been tentatively borne out by the observation that imidazole compounds are formed only early during growth of the culture and that later $C^{14}$ glycine is incorporated into a compound which is presumed to be the imidazolecarboxamide compound precursor.

It has also been assumed that the closure of carbon 2 would be at the ribotide level and therefore, not only the imidazoleribotide but also the riboside would accumulate. The tentative identification of the carboxamide pentotide and the isolation, purification and partial characterization of 5-$NH_2$-4-imidazolecarboxamide pentoside in the *E. coli* system would tend to support such a concept. The significance of these findings in relation to the mechanism of purine biosynthesis has been discussed.

Further studies on the mechanism of ring closure in the synthesis of inosinic acid in pigeon liver extracts have been reported. Homocysteine specifically stimulates the incorporation of $C^{14}$-formate into carbons 2 and 8 of the purine fraction of inosinic acid. ATP is necessary for incorporation of formate into inosinate. These findings have been discussed and a working scheme for the mechanism of activation of formate in pigeon liver has been suggested.[3]

Mrs. Hale Bumpus provided valuable assistance in the experimental studies reported.

[3] The suggested scheme for the mechanism of activation of formate represents the cooperative thinking of Dr. Paul Berg and the author while pursuing their independent problems. The author wishes to thank Dr. Berg for allowing him to quote his findings with homocysteine.

## ADDENDUM

Since the presentation of this paper the following pertinent information has been obtained. The carboxamide pentoside has been purified and identified as carboxamide riboside (26). Carboxamide ribotide has been more adequately identified in the *E. coli* system. Carboxamide riboside has been converted to carboxamide-5′-phosphoribotide either by yeast or pigeon liver extracts (27). This ribotide has been isolated in quantity, identified and shown to be rapidly converted to inosinic acid both by yeast and pigeon liver extracts. This reaction requires ATP, homocysteine, and formate. The role of citrovorum factor has not yet been ascertained as it is present in dialyzed extracts.

Figure 1 originally presented with riboside compounds early in the scheme has been brought to date to suggest early ribotide intermediates. Recent studies (GOLDTHWAIT, D. A., R. A. PEABODY AND G. R. GREENBERG, unpublished results) presented in part at the Federation Meetings in Chicago, April, 1953, have shown the accumulation in a partially fractionated pigeon liver system of aliphatic ribotides. The structures of these compounds have been only partially elucidated but may be represented schematically by *I* and *II* in figure 1. These compounds are converted to inosinic acid. Such a scheme would follow from a reaction of a base with a ribose-phosphate compound to directly form a ribotide such as suggested by the studies of Williams and Buchanan (5c).

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4. *American Society for Experimental Pathology.* Any American investigator who has contributed meritorious work in pathology within three years prior to his candidacy is eligible for membership. Candidates shall be nominated by two members and the nominations approved by the Council for election by Society members at a regular meeting. *Additional information and nomination forms may be obtained from* DR. C. C. ERICKSON, University of Tennessee, Institute of Pathology, Memphis 3, Tenn. (after July 1).

5. *American Institute of Nutrition.* Qualified investigators who have independently conducted and published meritorious original investigations in some phase of the chemistry or physiology of nutrition, and who have shown a professional interest for at least five years, are eligible for membership. Nominations shall be made and seconded by members of the Society and shall be submitted to the Council for recommendation to the Society at any regular meeting. *For nomination blanks and additional information address* Dr. J. M. ORTEN, Secretary, Wayne University College of Medicine, 1512 St. Antoine St., Detroit 26, Mich.

6. *American Association of Immunologists.* Any qualified person engaged in the study of problems related to the purpose of the Association may apply for membership. Candidates must be nominated by two members of the Association and applications must be accompanied by letters of recommendation, curriculum vitae and a list of reprints of publications. The Council shall determine eligibility and submit the names of eligible candidates to the membership at the annual meeting. *For further information and application blanks, address* DR. J. Y. SUGG, Cornell University Medical College, 1300 York Ave., New York 21, N. Y.